# Simian immunodeficiency virus (SIV) molecular epidemiology in non-human primates from West Africa

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät der

Universität Basel

von

**Sabrina Locatelli**

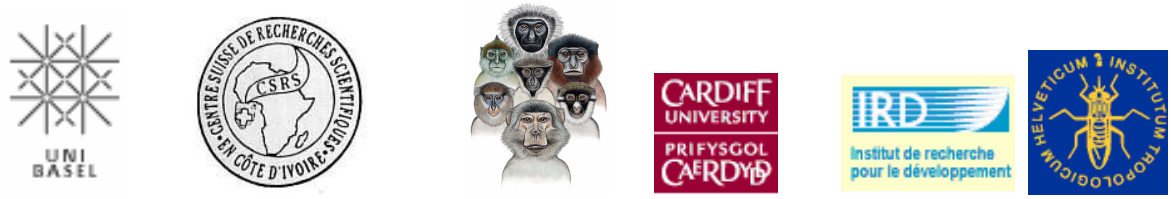
aus Muzzano, Tessin

Basel, 2008

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Herrn Prof. Dr. Marcel Tanner, Herrn PD Dr. Jakob Zinsstag, Frau Dr. Martine Peeters, Herrn Prof. Dr. Ronald Noë und Herrn PD Dr. C. Griot.

Basel, den 22. April 2008

Prof. Dr. Hans-Peter Hauri
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät



# Simian immunodeficiency virus (SIV) molecular epidemiology in non-human primates from West Africa

Accomplished with the supervision of

Dr. Martine Peeters (IRD, Montpellier and University of Montpellier 1, France)

and

PD Dr. Jakob Zinsstag (Swiss Tropical Institute, Basel)

at the University of Basel, Switzerland

***Ai miei genitori***

# Table of contents

# Acronyms

| | |
|---|---|
| **AIDS:** | Acquired Immunodeficiency Syndrome |
| **ANRS:** | Agence Nationale de Recherches sur le SIDA |
| **APOBEC3G:** | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G |
| **bp:** | base pairs |
| **BSA:** | Bovine Serum Albumin |
| **CDC:** | Center for Disease Control |
| **CD4/CD8:** | Cluster Designation 4/8 |
| **CNPRC:** | California National Primate Research Center |
| **CRF:** | Circulating Recombinant Form |
| **DNA:** | Deoxyribonucleic Acid |
| **EDTA:** | EthyleneDiamine Tetra-acetic Acid |
| **EIA:** | Enzyme ImmoAssay |
| **ELISA:** | Enzyme Linked ImmunoSorbent Assay |
| **FIV:** | Feline Immunodeficiency Syndrome |
| **gp:** | glycoprotein |
| **G6PDH:** | Glucose-6-Phosphate Dehydrogenase |
| **HIV:** | Human Immunodeficiency Virus |
| **HTLV:** | Human T-cell Leukemia Virus |
| **IRD:** | Institut de Recherche pour le Développement |
| **IgG:** | Immunoglobulin G |
| **kb:** | kilobase |
| **LIA:** | Line Immuno Assay |
| **LTR:** | Long Terminal Repeat |
| **MAP:** | Multiple Antigenic Peptides |
| **MAP:** | Maximum Posterior Probability |
| **MRCA:** | Most Recent Common Ancestor |
| **mtDNA:** | mitochondrial DNA |
| **NHP:** | Non Human Primate |
| **OPV:** | Oral Polio Vaccine |
| **ORF:** | Open Reading Frame |
| **PBMC:** | Peripheral Blood Mononuclear Cells |
| **PBS:** | Phosphate-Buffered Saline |
| **PCR:** | Polymerase Chain Reaction |

**PI:** Protease Inhibitor
**PP:** Posterior Probability
**RNA:** Ribonucleic Acid
**RT:** Reverse Transcriptase
**SFV:** Simian Foamy Virus
**SHIV:** Simian Human Immunodeficiency Virus
**SIDA:** Syndrome d'ImmunoDéficience Acquise
**SIV:** Simian Immunodeficiency Virus
**STD:** Sexually Transmitted Disease
**STLV:** Simian T-cell Leukemia Virus
**TAR:** Transactivation Response element
**TNP:** Taï National Park
**TRIM5α:** Tripartite motif-containing protein 5 alpha
**UNAIDS:** The Joint United Nations programme on HIV/AIDS
**V3**: Variable domain 3
**VL:** Viral Load
**WHO:** World Health Organization

# List of Figures

# List of Tables



# List of Pictures

# Acknowledgments

Writing this thesis has given me a chance to look back over the journey I have taken to get here. I look back with nostalgia and affection now, even if at times I doubted I was ever going to finish it. What is certain is that I could have never reached the destination without your emotional, intellectual, financial and logistical support. You were the fuel that powered my engine.

In chronological order: dedico questa tesi ai miei genitori che hanno creduto in me e approvato le mie scelte, non senza difficoltà certo, ma hanno saputo ascoltare i miei desideri e con coraggio accettare le trasferte della loro bambina in giro per il mondo. Hugs and thanks to my old friends Monica, Katya, Anna, Giuliano, Amanda, Eleonora, Francesca, Deborah, Miro, Claudio, Carine and Mireille, who during these years have put up with never ending stories and complaints, but also long silences without doubting our friendship.

This thesis has come about thanks to a series of events that took me first to Basel in 2002 and then to Côted'Ivoire. Despite having only worked a short time with Prof. Christophe Boesch, I would like to thank him for giving me the chance to discover the mystery and beauty of Taï National Park. All my love goes to Gerry who first encouraged me to pursue this career and who has followed my progress although from afar. Prof. Marcel Tanner, Director of the Swiss Tropical Institute, with his dynamic and proactive attitude, pushed me to enrol as a PhD student at Basel University even before I had a topic to investigate. I thank him for his faith in my potential and for his kindness and professional help. I am very grateful to Prof. Ronald Noë who decided to take me on his scientific boat and to introduce me to the Taï Monkey Project players. My greatest sympathy goes to Johannes Refisch and his natural goodwill and to the rest of the original crew of the project, Prof. Peter Nagel and Cécile Benetton.

Je remercie très sincèrement le Ministère de l'Environnement et de la Forêt, le Ministère de la Recherches Scientifique et de l'Enseignement Supérieur, le directeur de la Station du Centre de Recherche en Écologie à Taï (SRET) et le Projet Autonome pour le Parc National de Taï (PacPNT) de Côte d'Ivoire de m'avoir permis de travailler dans le Parc National de Taï. Je remercie également Simone et Olivier Girardin,

Guéladio et Aissatou Cissé pour leur support logistique et leurs conseils ainsi que les employés du Centre Suisse de Recherche Scientifique pour leur assistance et leur jovialité. Ma plus grande reconnaissance va aux assistants du Projet Singes de Taï, Bertin, Gérard, Ferdinand et Richard: sans leur connaissance de la forêt et de ses habitants, je n'aurais pas pu accomplir ce travail. Chapeau à leur courage et à leur détermination dans les moments très difficiles que leurs villages ont dû traverser pendant les périodes de crise politique. Un bisou au ciel à Benjamin. Un grand merci aussi aux étudiants du Projet Singes, Anne, Cécile et Anderson, pour leur amitié et leur sourire. Un merci spécial à Karim, ami et confident et à Jean-Claude pour son soutien chaleureux pendant mon séjour en Côte d'Ivoire. Je n'oublie pas les 'grands patrons', directeurs du Projet Singes de Taï, Dr. Klaus Zuberbühler, Dr. Scott McGraw et Dr. Johannes Refisch qui ont su mener le bateau à bon port. Je n'oublie pas non plus les membres du projet Chimpanzé de Taï, voisins de recherche, Camille, Nicaise, Sylvain, Jean-Baptiste, Touré et Grégoire. Une pensée à Gabriel et à sa famille. Un grand merci à Emmanuelle, ma compagne de forêt préférée, ma confidente, mon amie, mon équilibre et à Lee avec laquelle on a su rire jusqu'aux larmes de nos mésaventures. Enfin, un grand merci aux singes de Taï pour leur confiance !

I am deeply indebted to Andreas Moser for his friendship, his help in moving the financial machine, and for giving me the opportunity to learn from the molecular geneticists at The School of Biosciences in Cardiff. My deepest thanks to Prof. Michael W. Bruford and Dr. Amy Roeder for their supervision and teaching. A special thank to Yoshan for his caring bluntness and of course also to Fairus, Benoît, Gabriela, Haakon, Jo, Bowei, Mireille and Rhys for their academic and technical support, but mostly for their warm welcome, friendship and boundless dinner party energy.

Un immense merci au laboratoire Rétrovirus de Montpellier, où la fleur de mon travail a pu s'épanouir. Sans cette étape dans mon parcours, je n'aurais pas pu aboutir à cette thèse. Ma plus grande reconnaissance et mon plus grand respect vont au Dr. Martine Peeters et au Prof. Eric Delaporte pour leur inépuisable patience, leur support financier mais surtout pour leur encadrement académique et la rigueur enseignée. Je leur dois beaucoup, même si je n'ai pas toujours su le leur montrer. J'ai passé deux ans et demi dans ce laboratoire, et j'ai eu beaucoup en retour de chacun de ces 'pétales'.

Un c'est laissé emporter par le vent : Laurence chérie, j'aurais voulu partager plus que quelques années de ma vie avec toi, mais malheureusement le destin nous en a privé. À Florian, source de fous-rires - discussions stimulantes - disputes importantes, un énorme merci pour son soutien ainsi que toute ma gratitude pour l'instruction-support technique/intellectuel et la grande contribution au travail de laboratoire. 'Aux filles' Christelle et Fran pour leur assistance technique, leur encouragement, leur amitié et leur aide aussi en dehors du laboratoire. A Céline pour ses précieux conseils informatiques et techniques, à Nicole pour m'avoir initié au monde mystérieux du séquençage mais surtout pour sa sagesse et ses conseils. Aux secrétaires du laboratoires Monique, Audrey, Nadia et Coralie, pour leurs aides administratives et logistiques et leur bonne humeur ; à Cécile pour nos interminables discussions sur les primates humains et non-humains, à Halimatou et à Fabian pour leur amitié et leurs souvenirs d'Afrique, à Marjorie pour son aide dans la phase finale de cette rédaction, à Christian pour ses discussions socio-politico-épidémiologiques et enfin aux trois mousquetaires Camerounais: à Cyril pour avoir toujours su stimuler mon esprit critique, à Avelin, pour ses conseils techniques et son amitié et à Ahidjo, mon compagnon de bureau préféré, qui avec immense calme et disponibilité m'a appris beaucoup sur les secrets de la biologie moléculaire. Toujours au sein de l'IRD mais en dehors du laboratoire Rétrovirus, je dois ma gratitude au Dr. Bénédicte Lafay, pour son enthousiasme, sa contribution académique, spécialement dans le domaine de la phylogénie et pour les échanges fructueux au sein du projet; à Marie et Franck pour les soirées passées en leur compagnie et pour nos échanges scientifiques.

I would like to say how much I appreciated the scientific correspondence I exchanged with Nelson Ting, from the Anthropological department of the City University of New York.

At the STI, I am very grateful to PD Dr. Jakob Zinsstag for having warmly welcomed me in the frame of the 'Human and animal health' team, for his supervision, his support finding financial help and the ride on a "Swiss" camel! Equally, I thank the students and post-docs of this team, Jennifer, Lena, Rea, Bechir, Richard, Balako, Hung, Borna, Jalil and Thomas and also Peter for his help. I would also like to thank Christine Mensch, Margrith Slaui and Christine Walliser for their institutional support.

Most sincere thanks to Prof. Christian Griot and to Prof. Ronald Noë for their participation as co-referent and expert.

I acknowledge and thank for financial support the Commission for Research Partnerships with Developing Countries, Bern (KFPE), the Messerli foundation, Zürich, the Guggenheim-Schnurr Foundation, Basel, the Institut de Recherche pour le Dévéloppment (IRD), the Agence Nationale de Recherches pour le SIDA (ANRS) and the Jubilee Foundation, Basel. This thesis has been printed with the support of the “Dissertation Fonds” of the University of Basel.

# Summary

It is now scientifically proved that the human immunodeficiency viruses, HIV-1 and HIV-2, are the results of cross-species transmissions of the simian immunodeficiency viruses (SIV) that naturally infect non-human primates in sub-Saharan Africa. SIVsmm from sooty mangabeys (*Cercocebus atys atys*) is recognised as the progenitor of HIV-2, whereas SIVcpz from chimpanzees (*Pan troglodytes troglodytes*) and SIVgor from gorillas (*Gorilla gorilla gorilla*) in West-central Africa are the ancestors of HIV-1, the virus responsible for the AIDS (Acquired ImmunoDeficiency Syndrome) pandemic having already infected more than 60 million people.

Only non-human primates (NHPs) species from Africa are infected with SIVs. Serological evidence of SIV positivity has been shown for at least 40 of the 69 primate species found in Africa and this has been confirmed by DNA sequence analysis in 32 species.

Generally, SIVs do not induce an AIDS-like syndrome in their natural hosts, suggesting that they have been associated and evolved with their hosts over an extended period of time. However, if SIV crosses the species barrier, it may become pathogenic to the new host. The ancestors of HIV-1 and HIV-2 have crossed the species barrier to humans on multiple occasions, most likely through the contact with infected blood and tissues from primates hunted for bushmeat.

Bushmeat hunting has been a longstanding practice throughout sub-Saharan Africa, but the trade has increased in the last decades. Commercial logging has led to the construction of roads into remote forest areas and hunters are now making use of this newly developed infrastructure to penetrate previously inaccessible forests and capture and transport bushmeat to major city markets. Moreover, villages around logging concessions have become more densely populated; this has also increased the trade and consumption around these areas.

The socio-economic and environmental changes occurring combined with the growing genetic diversity and SIV prevalence among non-human primate populations, suggest that today, more than previously, the human population is exposed to SIVs. Bushmeat hunting is not limited to chimpanzees, gorillas or sooty mangabeys: the majority of

NHPs is represented by many *Cercopithecus* and *Colobus* species for example. It is therefore important to continue the search and the characterisation of new SIVs and to determine the prevalence of infection in the NHP, in order to better evaluate which of these SIVs represent a health threat for the human population.

The main goal of this thesis were

(i) to determine SIV infection and investigate its prevalence among different social groups of monkeys living in Taï National Park, Côte d'Ivoire, knowing that these NHPs are heavily hunted around this area

(ii) to determine SIV infection and prevalence and to characterise at a molecular level the SIV possibly infecting the red colobus species found in Abuko Nature Reserve, the Gambia. Two different subspecies of red colobus are found in the Gambia and in Côte d'Ivoire: this represented an opportunity to investigate whether different subspecies may harbour genetically different viruses and therefore to better understand the impact of geographical barriers on the evolution of SIV

(iii) to compare the molecular structure of SIVs infecting red and olive colobus, two sister species in the Colobinae subfamily

(iv) to determine if the chimpanzee subspecies found in West Africa is infected with a virus similar to that of the monkey species it preys upon, knowing that, to date, only the subspecies from West-central and East Africa have been found to be infected with SIV.

The majority of the results presented in this thesis have been obtained by analysing data collected with non-invasive methods. SIV infection has been determined in NHPs by detecting antibodies or by isolating viral sequences from freshly dropped faecal samples collected in the forests of Côte d'Ivoire and The Gambia. In order to be able to discriminate the faecal samples collected and, consequently, to reliably determine the prevalence of infection in a monkey group, the host has been genotyped by analysing the DNA extracted from the epithelial cells debris released from the

intestine. Host genotyping, antibody detection and isolation of viral RNA from faecal samples have become possible thanks to the improvement of conservation methods and DNA and RNA extraction techniques.

Wild-living non-human primate populations often live in inaccessible areas and tend to be wary of the presence of observers or display cryptic behaviour; the difficulty in sampling increases when the target species are arboreal primates exploiting the higher layers of the forest canopy. To mitigate these problems, we selected two field sites (Abuko Nature Reserve, The Gambia and Taï National Park, Côte d'Ivoire) where the primate populations were at least partly habituated by the presence of human observers and where behavioural-ecology studies have been conducted for more than 10 years.

In fact, SIV is transmitted mainly sexually, but possibly also vertically (from the mother to the offspring) and through biting or infection of open wounds. It is therefore important to consider factors such as mating system, patterns of dispersal, group size, average number of adult males in a group, polyspecific associations, etc. as parameters conducive to the transmission of the virus within or across groups and species.

In total, more than 300 faecal samples from two groups of western red colobus (*Piliocolobus badius badius*), from two groups of black-and-white colobus (*Colobus polykomos polykomos*), from three groups of olive colobus (*Procolobus verus*), from three groups of Diana monkeys (*Cercopithecus diana*), from one group of Campbell's monkeys (*Cercopithecus campbelli*), from one group of lesser-spot nosed monkeys (*Cercopithecus petaurista*) and from a group of greater spot-nosed monkeys (*Cercopithecus nictitans*) were collected near the western border of the Taï Forest, in Côte d'Ivoire, between March and July 2004.

To discriminate the faecal samples collected, 16 microsatellite loci were screened in these seven monkey species using cross-specific human markers. Microsatellites are di-tri-tetra-nucleotide tandem repeats, which length's variability is transmitted by Mendelian inheritance and can therefore be used in combination for individual discrimination. Between 25% to 37% of the primers used were informative and successfully and reliably amplified faecal extracted DNA from all species (Chapter 5).

*Colobus* and *Cercopithecus* samples were first tested for the presence of HIV cross-reactive antibodies using an immunoblotting assay and were found to be all negative or 'non interpretable'.

Subsequently, Reverse Transcriptase-Polymerase Chain Reactions (RT-PCRs) using universal as well as species-specific primers that target the *gag, pol* and *env* regions of the SIV genome were performed: only the western red colobus tested positive for SIV infection.

Among the inferred 53 adult individuals belonging to two neighbouring habituated groups, 14 tested SIVwrc (western red colobus) positive with a prevalence of 26%. Phylogenetic analysis of *pol* and *env* sequences revealed a low degree of viral genetic diversity in each group. The viral sequences obtained were generally clustering together according to their respective social group of origin. Conversely, the degree of viral genetic diversity between the two groups was higher.

Behavioural and demographic data collected previously from these communities indicate that western red colobus monkeys live in promiscuous multi-male societies, where females leave their natal group as sub-adults and where extra-group copulations or male immigration have been rarely observed. Phylogenetic data reflect these behavioural characteristics (Chapter 6).

The negative SIV results obtained for the other investigated species may reflect their social structure and mating system, but possibly also the difficulty of group monitoring, faecal sample collection coverage in the field as well as the long term conservation of viral RNA in the field and the sensitivity and specificity of SIV serological and molecular detection tools respectively (Chapter 9).

In parallel, sixteen faecal samples from sixteen individuals and two tissue samples from two carcasses of Temminck's red colobus monkeys (*Piliocolobus badius temminckii*) collected from the forest floor, between January and February 2005, in the Abuko Nature Reserve were analysed. None of the 16 faecal samples from Temminck's red colobus analysed by RT-PCR were positive. However, SIV infection was identified in one of the tissue samples, and phylogenetic analyses of partial *pol* and *env* sequences showed that this SIVwrc-*Pbt* virus strain is closely related to SIVwrc-*Pbb* strains from *P.b.badius* in the Taï Forest, suggesting that geographically separated subspecies can

be infected by closely related viruses. Molecular characterization and phylogenetic analysis of a SIVwrc-*Pbt* and two SIVwrc-*Pbb* full-length genomes, subsequently sequenced (Chapter 8), confirmed that SIVwrc-*Pbt* and SIVwrc-*Pbb* belong to a species-specific SIV lineage, although distantly related to the SIVlho lineage comprising SIVs from mandrills (*Mandrillus sphinx*), l'Hoest (*Cercopithecus lhoesti*) and sun-tailed monkeys (*Cercopithecus solatus*) (Chapters 7 and 8).

More recently, we characterised the full-length genome of the SIV infecting the olive colobus (SIVolc), by analysing a blood sample collected during a previous study also conducted in Taï National Park. Olive and western red colobus are sister taxon and results showed that SIVwrc and SIVolc form distinct lineages, but are closely related across their entire genome (Chapter 8).

These results confirmed the complex evolutionary history of primate lentiviruses, which has been driven by host-virus co-speciation, cross-species transmission and recombination events over an extended period of time. Genomic characterization of additional SIVs viruses from other Colobines is needed to better understand the ancestral phylogenetic relationship to SIVs from the l'Hoest lineage and whether recombination occurred between ancestors of these viruses.

Finally, 5 blood and tissue samples collected from 5 chimpanzee carcasses during a previous study conducted in Taï National Park, were analysed for SIV infection in this study. To date, no SIV has been isolated from this subspecies of chimpanzee (*Pan troglodytes verus*) found in West Africa. It is known that the chimpanzee hunts small monkeys and that SIVcpz, the precursor of HIV-1, isolated from the west-central chimpanzee subspecies (*Pan troglodytes troglodytes*), is a mosaic virus resulting from the recombination of viruses from different species of monkeys the chimpanzee preys upon. Following this line of reasoning, we tested universal as well as species-specific markers amplifying SIV infecting western red colobus, the favourite prey of chimpanzees from the Taï Forest. Serological tests conducted on these 5 samples showed a weak seropositivity in three of them. These results could not be confirmed by PCR. Whether this chimpanzee subspecies is not infected with SIV or whether it harbours a highly divergent virus not detected yet by the current molecular tools available remains to be determined (Chapter 10).

Overall, given the frequency of SIVwrc infection in the wild, the relative abundance of western red colobus, their cohabitation with other monkey species possibly carrying genetically different SIVs and the relatively high handling and consumption of their meat by chimpanzees and humans, the potential of simian to simian and simian to human cross-species transmissions cannot be excluded. The current HIV-1 pandemic is the result of a few cross-species transmissions. Many more could or may have already occurred, but remain undetected by the current serological or molecular tools available, or simply because people at risk may live in non accessible isolated regions of Africa.

Studies are needed to determine whether transmissions of SIVs from primates other than chimpanzees and mangabeys have already occurred - by developing even more sensitive serological tests and specific molecular tools - and to assess possible clinical outcomes associated with these infections. Newly discovered virus strains would need to be tested for their capability of infecting human cells by measuring their growth and cytotoxic potential.

Results from these studies will yield critical insights into the circumstances and factors that govern SIV cross-species transmission and thus allow a better understanding of the risk of human infection by these viruses. Moreover, due to the increasing prevalence of HIV in African rural areas, recombination between newly introduced SIVs and circulating HIVs may pose an additional risk for the outbreak of a novel HIV epidemic.

# Zusammenfassung

Es ist heute unbestritten, dass die Humanimmunschwächeviren HIV-1 und HIV-2 das Resultat einer Artschranken überschreitenden Übertragung von natürlich vorkommenden Immunschwächeviren nicht-menschlicher Primaten (SIV: Simian Immunodeficiency Viruses) in Subsahara-Afrika sind. Das SIVsmm der Russmangaben (*Cercocebus atys atys*) gilt als Ursprungsform von HIV-2, während das SIVcpz der Schimpansen (*Pan troglodytes troglodytes*) und das SIVgor der Gorillas (*Gorilla gorilla gorilla*) im westlichen Zentralafrika als die Ursprungsformen von HIV-1 angesehen werden. HIV-1 ist das Virus, welches für die HIV/Aids Pandemie verantwortlich ist, die weltweit über 60 Millionen Menschen betrifft.

Nur die Altweltaffen Afrikas sind mit SI-Viren infiziert und bei mindestens 39 der bekannten 69 Arten gibt es serologisch positive Befunde, die bei 32 Arten zusätzlich durch Sequenzanalyse bestätigt worden sind.

Normalerweise verursachen SI-Viren bei ihren natürlichen Wirten keine AIDS-ähnlichen Syndrome, was nahe legt, dass sie bereits über lange Zeiträume mit ihren Wirten assoziiert sind und zusammen evoluiert haben. Hingegen kann das SI-Virus, wenn es eine Artschranke überspringt, für den neuen Wirt äusserst pathogen sein. Die Ursprungsformen von HIV-1 und HIV-2 haben die Artbarriere zum Menschen mehrfach überwunden, vermutlich vorwiegend durch den Kontakt mit infiziertem Blut oder Gewebe von Primaten, die als Wildfleisch (*Bushmeat*) gejagt worden sind.

*Bushmeat* hat in Subsahara-Afrika eine lange Tradition. In den letzten Jahren ist der *Bushmeat*-Handel aber stark angestiegen. Die grossflächige industrielle Nutzung der Holzreserven hat dazu geführt, dass immer mehr Strassen in abgelegene Waldgebiete hineingetrieben werden und dadurch Jäger in bisher unzugängliche Gebiete eindringen können. Diese Wilderer nutzen zudem die Strassen und die darauf verkehrenden Holztransporter, um das Fleisch auf die Märkte der grossen Städte zu transportieren. Auch ziehen die Dörfer rund um die Holzschlag-Konzessionen immer mehr Menschen an, so dass überall neue lokale Märkte entstehen.

Die Kombination dieser sozioökonomischer und ökologischer Veränderungen auf der einen und der zunehmenden genetischen Vielfalt der SI-Viren und deren hohe Prävalenz unter den nicht-humanen Primatenpopulationen auf der anderen Seite legen nahe, dass die menschliche Population heute in viel stärkerem Masse als früher SI-Viren ausgesetzt ist. Die Jagd nach *Bushmeat* beschränkt sich nicht nur auf Schimpansen, Gorillas und Russmangaben; die Mehrzahl der gejagten nichtmenschlichen Primaten sind Arten der Gattungen *Colobus* und *Cercopithecus*. Es ist deshalb von grosser Wichtigkeit, die Suche nach neuen SI-Viren, ihre Charakterisierung und die Bestimmung ihrer Prävalenz unter frei lebenden nicht-humanen Primaten weiterzuführen, um die Früherkennung potentiell humanpathogener Viren sicherzustellen.

Die Hauptziele dieser Dissertation waren

(i) die allgemeine SIV Infektionsratio sowie die Prävalenz bei habituierten sozialen Gruppen verschiedener Affenarten imTaï Nationalpark zu untersuchen (insbesondere da diese nicht-humanen Primaten ausserhalb des Parks stark bejagt werden)

(ii) bei Roten Stummelaffen im Abuko Naturreservat in Gambia die SIV Prävalenz zu bestimmen und das Virus auf molekularer Ebene zu charakterisieren (Die Roten Stummelaffen in Gambia gehören einer anderen Unterart an als jene der Elfenbeinküste. Dies bot eine Gelegenheit zu untersuchen, ob verschiedene Unterarten Träger unterschiedlicher Viren sind und einen tieferen Einblick in die Auswirkungen von geografischen Barrieren auf die Evolution von SI-Viren gewinnen zu können.)

(iii) die molekulare Struktur der SI-Viren der Roten und der Grünen Stummelaffen, zweier nahe verwandter Arten der Unterfamilie *Colobinae*, zu vergleichen, und

(iv) zu untersuchen, ob die westafrikanische Schimpansen-Unterart (*Pan troglodytes verus*) mit einem ähnlichen Virus infiziert ist wie die Affenarten, die sie jagt (bis heute konnte dies erst bei den zentral- und ostafrikanischen Unterarten nachgewiesen werden).

Ein Grossteil der in dieser Dissertation präsentierten Resultate wurde durch die Analyse von Proben und Daten erarbeitet, die mit nicht-invasiven Methoden gesammelt worden sind. Eine SIV-Infektion wurde durch die Bestimmung von Antikörpern oder die Isolation von viralen Sequenzen in frisch defäkierten Stuhlproben festgestellt. Die Proben wurden während der direkten Beobachtung der Gruppen und der defäkierenden Individuen vom Waldboden aufgesammelt. Um die Proben zuverlässig zuweisen und damit auch die Prävalenz in der Gruppe bestimmen zu können, wurden die Wirte durch DNS Analyse der mit dem Stuhl ausgeschiedenen Darmepithelzellen-Fragmente genotypisiert. Wirt-Genotypisierung, Antikörperbestimmung und die Isolierung viraler RNS aus Stuhlproben wurde erst möglich dank neuer Konservierungsmethoden im Feld und durch die Verfeinerung der DNS- und RNS-Extraktionstechniken im Labor.

Frei lebende Primatenpopulationen leben oft in unzugänglichen Gebieten, sind scheu und misstrauisch gegenüber Beobachtern oder favorisieren eine sehr versteckte Lebensweise. Die Probenaufnahme wird zusätzlich erschwert, wenn die untersuchte Art baumbewohnend ist und bevorzugt die höheren Kronenbereiche des Waldes nutzt. Um den Einfluss dieser Probleme möglichst gering zu halten, haben wir zwei Untersuchungsgebiete gewählt (Abuko Naturreservat, Gambia, und Taï Nationalpark, Elfenbeinküste), in welchen die Primatenpopulationen zumindest teilweise schon habituiert und an die Anwesenheit von Beobachtern gewöhnt waren und wo bereits seit mehr als zehn Jahren Verhaltensuntersuchungen durchgeführt worden sind.

SI-Viren werden hauptsächlich sexuell, möglicherweise aber auch vertikal (von Mutter zu Kind) sowie durch Bisse und die Infektion offener Wunden übertragen. Es ist daher unumgänglich, Faktoren wie Fortpflanzungsysteme, Ausbreitungs- und Abwanderungsmuster, Gruppengrösse, durchschnittliche Zahl adulter Männchen in einer Gruppe, zwischenartliche Vergesellschaftungen, etc. als wichtige Parameter für die intra- und interspezifische Übertragung des Virus zu berücksichtigen.

Rund 300 frisch defäkierte Stuhlproben von zwei Gruppen des westlichen Roten Stummelaffen (*Piliocolobus badius badius*), zwei Gruppen des Bärenstummelaffen (*Colobus polykomos polykomos*), drei Gruppen des Grünen Stummelaffen (*Procolobus verus*), drei Gruppen der Diana-Meerkatze (*Cercopithecus diana*), einer Gruppe der Campbell-Meerkatze (*Cercopithecus campbelli*), einer Gruppe der Kleinen

Weissnasenmeerkatze (*Cercopithecus petaurista*) sowie einer Gruppe der Grossen Weissnasenmeerkatze (*Cercopithecus nictitans*) wurden von März bis Juli 2004 auf dem Waldboden des Taï Nationalparks, Elfenbeinküste, gesammelt.

Um die gesammelten Stuhlproben zuweisen zu können, untersuchten wir 16 Mikrosatelliten-Loci dieser sieben Affenarten mit Hilfe von cross-spezifischen Humanmarkern. Mikrosatelliten sind di-tri-tetra-nucleotide Tandemwiederholungen, deren Längenvariabilität nach Mendel'schen Vererbungsregeln weitergegeben werden und die daher in ihrer Gesamtheit zur Individualerkennung eingesetzt werden können. 25-37% dieser Primer konnten erfolgreich und zuverlässig DNA aus den Stuhlproben aller Arten amplifizieren (Kapitel 5).

Die *Colobus* und *Cercopithecus* Proben wurden zuerst mit einer Immunoblotting-Methode auf die Anwesenheit von HIV cross-reaktiven Antikörpern getestet. Sie reagierten alle 'negativ' oder 'nicht interpretierbar'.

Anschliessend wurden auch Reverse Transcriptase Polymerase Kettenreaktionen (RT-PCR). Untersuchungen durchgeführt, sowohl mit universellen wie auch mit artspezifischeren Primern, die auf die *gag*, *pol* und *env* Sequenzen des SIV Genoms abzielen. Einzig der westliche Rote Stummelaffe testete positiv für SIV.

Von den 53 identifizierten Individuen aus zwei benachbarten habituierten Gruppen testeten 14 SIVwrc (westlicher Roter Stummelaffe) positiv, mit einer Prävalenz von 26%. Mit wenigen Ausnahmen zeigte die phylogenetische Analyse der *pol* und *env* Sequenzen einen niedrigen Grad genetischer Diversität innerhalb der Gruppen und eine allgemeine virale Häufung, die auf die jeweilige soziale Ursprungsgruppe hinweist. Allerdings fanden wir einen höheren Grad genetischer Diversität zwischen den beiden Gruppen.

Frühere ethologische und demografische Untersuchungen an diesen beiden Gruppen zeigten, dass westliche Rote Stummelaffen in promiskuitiven Vielmännergesellschaften leben, in welchen die Weibchen ihre Geburtsgruppen als Subadulte verlassen und wo Kopulationen ausserhalb der Gruppen oder die Einwanderung fremder Männchen nur selten beobachtet worden sind. Unsere phylogenetischen Daten scheinen diese Verhaltens-Charakteristika zu bestätigen (Kapitel 6).

Die negativen SIV Resultate bei anderen untersuchten Arten mögen mit deren Sozialstruktur und Fortpflanzungssystem zusammenhängen, aber ebenso mit der begrenzten Zahl der im Feld gesammelten Stuhlproben wie auch der fehlenden Spezifität der serologischen und molekularen SIV Nachweismittel (Kapitel 9).

Gleichzeitig untersuchten wir 16 Stuhlproben von sechzehn frei lebenden Tieren und zwei Gewebeproben von zwei Kadavern von Roten Temminck-Stummelaffen (*Piliocolobus badius temminckii*), die im Januar / Februar 2005 auf dem Waldboden des Abuko Naturreservats gefunden worden sind. Keine der 16 Stuhlproben testete RT-PCR-positiv. Eine SIV Infektion konnte nur bei einer Gewebeprobe nachgewiesen werden. Phylogenetische Analysen der partiellen *pol* und *env* Sequenzen zeigten, dass die neue SIVwrc-Pbt Linie eng mit den SIVwrc-Pbb Linien von *P.b.badius* im Taï Nationalpark (Elfenbeinküste) verwandt ist, was nahe legt, dass selbst geografisch getrennte Unterarten mit einem eng verwandten Virus infiziert sein können. Die anschliessend durchgeführte molekulare Charakterisierung und phylogenetische Analyse einer SIVwrc-Pbt und zweier SIVwrc-Pbb Genome über deren ganze Länge (Kapitel 8) bestätigten, dass SIVwr-Pbt und SIVwrc-Pbb einer artspezifischen Linie angehören, auch wenn sie entfernt mit der SIVlho Linie verwandt ist, welche SI-Viren des Mandrills (*Mandrillus sphinx*), der Vollbart- (*Cercopithecus lhoesti*) wie auch der Sonnenschwanz-Meerkatze (*Cercopithecus solatus*) umfasst.

Erst kürzlich charakterisierten wir das gesamte Genom des SI-Virus des Grünen Stummelaffen (SIVolc), indem wir Blutproben analysierten, die während einer früheren Studie (ebenfalls im Taï Nationalpark) genommen worden waren. Grüne und westliche Rote Stummelaffen sind Schwesterarten und die Resultate zeigen, dass SIVwrc und SIVolc zwar eigenständige Linien darstellen, aber dennoch über ihr ganzes Genom eng verwandt sind (Kapitel 8).

Diese Resultate bestätigen, dass die komplexe evolutive Geschichte der Primaten-Lentiviren über einen langen Zeitraum durch Wirt-Virus-Co-Speziation, interspezifische Übertragung und Rekombination bestimmt worden ist. Die Genom-Charakterisierung der SI-Viren weiterer *Colobinae* ist notwendig, um die Entwicklungsgeschichte und die phylogenetischen Verwandtschaften mit SI Viren der

l'Hoesti Linie besser zu verstehen und um nachweisen zu können, ob Rekombinationen zwischen den Vorfahren der SIVwrc und der SIVlho Linie stattgefunden haben.

Schliesslich wurden auch 5 Blut- und Gewebeproben, die von 5 Schimpansenkadavern während einer früheren Studie im Taï Nationalpark genommen worden sind, auf eine SIV-Infektion untersucht. Bisher konnte kein SIV in dieser westafrikanischen Schimpansen-Unterart (*Pan troglodytes verus*) nachgewiesen werden. Es ist bekannt, dass Schimpansen Kleinaffen jagen und dass SIVcpz, die Ursprungsform von HIV-1, das die Schimpansen-Unterart aus dem westlichen Zentralafrika (*Pan troglodytes troglodytes*) infiziert, ein Mosaikvirus ist, das aus einer Rekombination anderer Viren entstanden ist, die von verschiedenen Affenarten stammen, welche von den Schimpansen gejagt werden. Daher testeten wir sowohl universelle wie auch artspezifische Marker, welche die SI-Viren des westlichen Roten Stummelaffen, der bevorzugten Beute der Taï-Schimpansen, amplifizieren. Serologische Tests an allen fünf Proben ergaben nur drei schwach seropositive Reaktionen. Diese Resultate konnten mit PCR nicht bestätigt werden. Ob diese Unterart nicht mit SIV infiziert ist oder ob es ein hochdivergentes Virus beherbergt, das wir mit den verfügbaren molekularen Nachweismitteln noch nicht identifizieren können, muss noch weiter untersucht werden (Kapitel 10).

Rote Stummelaffen erfüllen viele Voraussetzungen, die sie zum Ausgangspunkt einer weiteren interspezifischen Affen-Affen oder Affen-Menschen Übertragung machen könnten: sie sind relativ häufig, ihre SIVwrc Prävalenz in der frei lebenden Population ist hoch, sie leben mit Affenarten zusammen, die möglicherweise Träger anderer SI-Viren sind, und sie werden sowohl von Schimpansen wie auch von Menschen oft gegessen.

Die aktuelle HIV1-Pandemie ist die Folge einiger weniger interspezifischer Übertragungen und viele weitere mögen schon stattgefunden haben, nur dass wir sie mit den zur Zeit verfügbaren serologischen und molekularen Methoden noch nicht nachweisen können, oder einfach weil die betroffenen Menschen in nicht zugänglichen, isolierten Regionen Afrikas leben.

Weitere Untersuchungen sind nötig um festzustellen, ob bereits weitere Übertragungen von SIV von anderen Primaten als Schimpansen und Mangaben

stattgefunden haben. Wir müssen noch empfindlichere serologische Tests und spezifische molekulare Nachweismittel entwickeln und versuchen, klinische Manifestationen dieser potentiellen neuen Infektionen möglichst früh zu erkennen. Neu entdeckte Virus-Linien müssen auf ihre Pathogenität gegenüber menschlichen Zellen untersucht werden, indem wir ihr Wachstum und ihr cytopathisches bzw. cytotoxisches Potential bestimmen. Solche Studien könnten uns entscheidende Einsichten bringen in die Umstände und Faktoren, welche die interspezifische Übertragung von SIV steuern und uns damit ein besseres Verständnis geben, wann und wie sich Menschen mit diesen Viren anstecken können.

Wegen der ansteigenden Prävalenz von HIV in den ländlichen Gebieten, kann eine Rekombination zwischen neu eingeführten SI-Viren und bereits zirkulierenden HI-Viren ein zusätzliches Risiko darstellen für den Ausbruch einer ‚neuen' HIV Epidemie. Deshalb ist es unumgänglich, die Identifikation von SI-Viren in wild lebenden Primaten weiter zu führen und nach Indikatoren zu suchen, welche Pathogene ein potentielles Risiko für den Menschen darstellen.

# Résumé

Il est désormais reconnu que les virus de l'immunodéficience humaine, VIH-1 et VIH-2, sont issus de la transmission inter-espèce des virus de l'immunodéficience simienne (SIV) infectant naturellement les primates non-humains en Afrique sub-saharienne. Le SIVsmm infectant les cercocèbes enfumés (*Cercocebus atys atys*) est à l'origine du VIH-2, tandis que le SIVcpz infectant les chimpanzés (*Pan troglodytes troglodytes*) et le SIVgor des gorilles des plaines (*Gorilla gorilla gorilla*) dans la région centre-ouest de l'Afrique sont les ancêtres du VIH-1, responsable de la pandémie du VIH/SIDA (Syndrome de l'Immunodéficience Acquise), ayant infecté à ce jour plus de 60 millions de personnes.

Seuls les primates du continent africain sont infectés par le SIV. Des résultats sérologiques positifs ont été mis en évidence chez au moins 40 espèces de primates sur les 69 présentes en Afrique et, pour 32 d'entre elles, les analyses moléculaires ont confirmé la présence de SIV.

Les SIVs n'induisent pas, en général, chez leurs hôtes de syndrome d'immunodéficience, ce qui suggère une longue association et coévolution entre le virus et son hôte. En revanche, en franchissant la barrière des espèces, le SIV peut devenir pathogène pour le nouvel hôte. Les ancêtres des VIH-1 et VIH-2 ont franchi cette barrière vers l'homme à plusieurs occasions, probablement par le contact avec le sang et les tissus infectés des primates chassés.

La consommation de viande de brousse a toujours été une pratique courante en Afrique sub-saharienne, mais son commerce a augmenté durant les dix dernières années. Les industries du bois ont favorisé l'ouverture de nouvelles routes dans des régions forestières et les braconniers pénètrent aujourd'hui des parties de forêt qui étaient précédemment inaccessibles et utilisent les nouvelles infrastructures développées pour la capture et le transport de la viande de brousse jusqu'aux marchés des plus grandes villes. Le développement et l'expansion des villages autour des concessions forestières ont contribué à une demande croissante des besoins protéiques des habitants et ont créé de nouvelles filières de commerce.

Les changements socio-économiques et environnementaux, combinés à la variété génétique et la prévalence des SIVs chez les primates non-humains, suggèrent qu'aujourd'hui, plus que jamais, la population humaine est exposée aux lentivirus de primates. La chasse n'est pas limitée aux chimpanzés, aux gorilles et aux mangabeys enfumés mais elle est étendue à plusieurs espèces de cercopithèque, de colobes, etc. Il est donc très important de continuer l'identification de nouveaux lentivirus et d'en mesurer la prévalence chez les primates sauvages afin de pouvoir évaluer quels virus pourraient représenter un risque pour la population humaine.

Le but de cette thèse était de

(i) évaluer la présence d'infections à SIV et d'étudier sa prévalence chez différents groupes sociaux de singes habitués du Parc National de Taï, Côte d'Ivoire, en sachant que la chasse est une pratique courante dans cette région

(ii) déterminer la prévalence et de caractériser au niveau moléculaire le SIV des colobes bai de la Réserve Naturelle d'Abuko, Gambie. Ces colobes bai appartiennent à une sous-espèce différente de celle présente en Côte d'Ivoire : ceci était une opportunité nous permettant de déterminer si des sous-espèces différentes peuvent être infectées par des virus génétiquement différents et si les barrières géographiques ont une quelconque influence sur l'évolution du SIV

(iii) comparer la structure moléculaire des SIVs du colobe bai et du colobe vert, deux espèces différentes mais apparentées

(iv) déterminer si la sous-espèce de chimpanzé de l'Afrique de l'ouest puisse être infectée par un virus génétiquement proche à celui des singes dont il se nourrit, en sachant que, seuls chez les sous-espèces de chimpanzé d'Afrique centrale et de l'est le SIV a été isolé.

La majorité des résultats de cette thèse a été obtenue grâce à l'analyse de données collectées avec des méthodes non-invasives. L'infection à SIV a été déterminée par détection d'anticorps et par isolement d'ADN et d'ARN viraux extraits à partir de matériel fécal de l'hôte collecté au sol dans les forêts de la Côte d'Ivoire et de la Gambie. Afin de déterminer la prévalence de l'infection à SIV, le nombre

d'échantillons issus d'individus différents récoltés a été discriminé en observant les animaux, mais surtout par genotypage de l'ADN de l'hôte à partir des cellules épithéliales intestinales présentes dans les fèces. Ces procédés ont été possibles grâce à l'amélioration des techniques de conservation du matériel génétique et d'extraction d'ADN, d'ARN et d'anticorps à partir de matériel partiellement dégradé.

Les populations naturelles de primates vivent souvent dans des régions peu accessibles, sont difficilement apprivoisables et présentent des comportements souvent cryptiques. Les espèces étudiées, colobes et cercopithèques, sont arboricoles et vivent dans les parties les plus hautes de la canopée forestière, ce qui rend difficile leur étude.

Ceci est la raison pour laquelle nous avons choisi deux sites d'étude (Réserve Naturelle d'Abuko, Gambie et Parc National de Taï, Côte d'Ivoire) où les populations de primates sont au moins partiellement habituées à la présence d'observateurs humains et où des études comportementales et écologiques ont été menées pendant plus de dix ans.

En effet, le SIV est transmis principalement par voie sexuelle, mais probablement aussi par voie verticale (de la mère à l'enfant) ou par des morsures. Il était donc important de considérer les aspects comportementaux et écologiques, tels le système de reproduction, le mode de dispersion, la taille du groupe, le nombre moyen de mâles adultes par groupe, les associations polyspécifiques, ainsi que la vulnérabilité de chaque espèce face à la pression de chasse. Tous ces facteurs peuvent favoriser la transmission intra- et inter-espèces du virus.

Plus de 300 échantillons fécaux provenant de deux groupes sociaux de colobes bai (*Piliocolobus badius badius*), deux groupes sociaux de colobes magistrats (*Colobus polykomos polykomos*), trois groupes de colobes verts (*Procolobus verus*), trois groupes de cercopithèques Diane (*Cercopithecus diana*), un groupe de Mone de Campbell (*Cercopithecus campbelli*), un groupe de cercopithèques à nez blanc (*Cercopithecus petaurista*) et enfin d'un groupe de cercopithèques hocheurs (*Cercopithecus nictitans*) ont été récoltés. Ces différents échantillons ont été collectés près de la frontière ouest de la forêt de Taï en Côte d'Ivoire, entre mars et juillet 2004.

Les échantillons fécaux ont été identifiés par l'observation sur le terrain mais aussi en génotypant leur ADN. Seize microsatellites mis au point pour l'homme ont été sélectionnés pour l'étude de ces 7 espèces de singes. Les microsatellites sont des di-tri-tétra nucléotides répétés en tandem de longueur variable, transmis par héritage Mendélien pouvant donc être utilisés en combinaison pour la discrimination d'individus. Entre 25% et 37% de ces 16 marqueurs génétiques ont été amplifiés avec succès selon l'espèce (Chapitre 5).

Les échantillons de colobes et de cercopithèques ont été testés pour la présence d'anticorps réagissant de façon croisés avec des antigènes VIH. Les résultats se sont révélés être négatifs ou non interprétables.

Ensuite, des réactions de transcriptase inverse et des réactions de polymérisation en chaîne (RT-PCR) ont été effectuées, en utilisant à la fois des oligonucléotides universels mais aussi des oligonucléotides spécifiques à l'espèce virale ciblant les régions *gag*, *pol*, et *env* du génome SIV. Seules les analyses sur les échantillons de colobes bai ont mis en évidence des infections lentivirales.

Quatorze sur 53 colobes bai, appartenant à deux groupes voisins, dont les individus ont été déterminés par l'analyse des microsatellites, sont infectés par SIVwrc (Western Red Colobus) avec une prévalence de 26% chez les individus échantillonnés. Les analyses phylogénétiques des séquences *pol* et *env* ont révélé un degré peu élevé de diversité génétique intra-groupe et en général un groupement viral qui correspond au groupe social d'origine. En revanche, nous avons une plus grande diversité génétique entre les deux groupes.

Les données comportementales et démographiques collectées précédemment sur ces communautés ont indiqué que les colobes bai vivent dans une société multi-mâles multi-femelles, où les femelles émigrent de leur groupe natal au stade de sub-adulte et où les reproductions extra-groupe ou la dispersion des mâles n'ont été que rarement observées. Les résultats phylogénétiques obtenus sur ces SIV semblent refléter certaines de ces caractéristiques comportementales (Chapitre 6).

Les résultats négatifs obtenus chez les autres espèces pourraient résulter d'une structure sociale et de reproduction différente, mais peut-être aussi de notre difficulté dans le suivi des groupes. En effet, le nombre d'individus échantillonnés, ainsi que la

conservation de l'ARN viral à long terme et la sensibilité et la spécificité des méthodes de détection, respectivement sérologiques et moléculaires, ont pu engendrer les résultats négatifs observés (Chapitre 9).

Parallèlement, seize échantillons fécaux, appartenant à seize individus, et deux échantillons de tissu, prélevés sur deux carcasses de colobes bai (*Piliocolobus badius temminckii*), ont été récoltés dans la Réserve Naturelle d'Abuko, Gambie, entre janvier et février 2005. Aucun des seize échantillons fécaux analysés dans cette deuxième étude n'était positif en utilisant la méthode de RT-PCR. Le SIV a pu être identifié uniquement chez un des deux échantillons de tissu. Les analyses phylogénétiques de séquences partielles des gènes *pol* et *env* ont montré que le virus SIVwrc-*Pbt* est très proche des souches de SIVwrc-*Pbb* de *P.b.badius* de la forêt de Taï en Côte d'Ivoire. Ceci suggère que des sous-espèces géographiquement séparées sont infectées par des virus apparentés (Chapitre 7).

La caractérisation moléculaire et les analyses phylogénétiques du génome complet de SIVwrc-*Pbt* et de deux autres génomes complets séquencés plus tardivement, ont confirmé que SIVwrc-*Pbt* et SIVwrc-*Pbb* appartiennent à une lignée de SIV spécifique à l'espèce, bien que apparentée à la lignée de SIV des cercopithèques de l'Hoest (SIVlho), des cercopithèques à queue dorée (SIVsun) et des mandrills (SIVmnd-1) (Chapitres 7 et 8).

Nous avons plus récemment caractérisé le génome complet de SIV chez les colobes verts (SIVolc) à partir d'un échantillon de sang prélevé au cours d'une étude précédente au Parc National de Taï. Le colobe vert est un taxon très proche des colobes bai. Les analyses de ces virus ont montré que le SIVwrc et le SIVolc appartiennent à des lignées distinctes mais apparentées sur la totalité du génome (Chapitre 8).

Ces résultats confirment une histoire évolutive complexe des lentivirus de primates, lesquels ont co-évolué avec leurs hôtes par des processus différents de transmission entre espèces et d'événements de recombinaison. Il faudrait aussi caractériser des virus additionnels de colobes pour mieux comprendre leur relation phylogénétique ancestrale avec les SIV de la lignée SIVlho et établir s'il y a eu une recombinaison entre ancêtres de ces virus.

Enfin, des tests sérologiques sur 5 échantillons de tissus prélevés sur des carcasses de chimpanzés collectés pour une étude précédente au Parc National de Taï, ont montré des résultats faiblement positifs pour 3 d'entre eux. Jusqu'à présent aucun SIV n'a été identifié chez la sous-espèce de chimpanzés (*Pan troglodytes verus*) habitant les forêts de l'Afrique de l'Ouest. Le chimpanzé chasse fréquemment des espèces de petits singes et il a été démontré que le SIVcpz, le précurseur du VIH-1, isolé chez la sous-espèce de chimpanzé habitant l'Afrique Centrale, est un virus mosaïque, issu de la recombinaison de virus isolés à leur tour chez différentes espèces de singes chassés par les chimpanzés. Selon cette logique, les 5 échantillons ont été testés avec des amorces virales universelles ainsi que spécifiques, amplifiant par exemple le SIVwrc chez le colobe bai, une des proies préférées du chimpanzé de la forêt de Taï. Les résultats sérologiques n'ont pas pu être confirmés par PCR. Il reste encore à confirmer si ces résultats reflètent une réelle absence de SIV chez cette sous-espèce ou si le virus qui l'infecte est assez divergent pour échapper à la détection par les outils moléculaires courants (Chapitre 10).

Dans l'ensemble, étant donnés la fréquence d'infection à SIVwrc dans la nature, la relative abondance des colobes bai, le partage du même habitat avec d'autres espèces de singes infectées par des SIV génétiquement différents et étant donné la chasse et la consommation considérable de cette espèce par les chimpanzés et l'homme, toutes les conditions sont réunies pour une transmission singe-singe ou singe-homme. La pandémie actuelle de VIH-1 est le résultat d'un nombre limité de transmissions entre espèces mais d'autres transmissions auraient déjà pu ou pourraient se produire sans être détectées avec les outils courants.

D'autres études sont nécessaires pour déterminer si la transmission de SIVs, excepté ceux qui infectent les cercocèbes enfumés ou les chimpanzés, aurait pu déjà se produire, en développant des tests sérologiques encore plus sensibles et des outils moléculaires encore plus spécifiques. Il faudrait aussi être en mesure d'évaluer les résultats cliniques associés à ces infections. Les souches nouvellement découvertes devraient être testées pour leur capacité à infecter des cellules humaines en mesurant leur taux de croissance et leur potentiel cytotoxique.

Les résultats issus de ces études nous fourniront un aperçu critique des facteurs qui gouvernent la transmission du SIV entre espèces et nous permettront de mieux comprendre les risques encourus par l'homme. De plus, au vue de l'augmentation de la prévalence du VIH dans les zones rurales d'Afrique et de la possibilité de recombinaison entre des SIVs et des VIH circulants, un risque d'émergence de nouvelles épidémies existe.

# Riassunto

È ormai scientificamente provato che i virus dell'immunodeficienza umana, HIV-1 e HIV-2, sono il risultato di trasmissioni di virus dell'immunodeficienza delle scimmie (SIV: "Simian Immunodeficeincy Virus") presente naturalmente in alcune specie di primati non umani dell'Africa sub Sahariana. Il cercocebo grigio (*Cercocebus atys atys*) è infetto da SIVsmm. Questo virus è riconosciuto come il progenitore del HIV-2, mentre SIVcpz nello scimpanzé (*Pan troglodytes troglodytes*) e SIVgor nel gorilla (*Gorilla gorilla gorilla*) dell'Africa centro-occidentale sono i progenitori dell'HIV-1, il virus responsabile della pandemia di AIDS (Aquired ImmunoDeficiency Syndrome = Sindrome da Immunodeficienza Acquisita) che ha sino ad oggi infettato più di 60 milioni di persone nel mondo.

Numerose specie di primati sono infette da questo virus ma unicamente quelle provenienti dal continente africano. Le analisi serologiche effettuate sino ad oggi hanno potuto confermare la presenza di SIV in 40 delle 69 specie di primati conosciute in Africa e in 32 specie vi è stata un'ulteriore conferma grazie all'analisi di sequenze di DNA.

Il virus SIV non induce una malattia tipo-AIDS nel suo ospite naturale. Questo ci suggerisce che virus e ospite si siano associati e abbiano co-evoluto per un lungo periodo di tempo. Se invece questo virus attraversa la barriera delle specie, potrebbe diventare patogeno per il nuovo ospite che lo contrae. I precursori dell'HIV-1 e HIV-2 hanno attraversato la barriera della specie umana in molteplici occasioni, più probabilmente attraverso il contatto con sangue e tessuti infetti di primati catturati durante la caccia.

Negli ultimi decenni, in Africa, la caccia e il commercio di selvaggina sono aumentati considerevolmente a causa specialmente dell'espansione dell'industria del legno, la quale favorisce la costruzione di strade verso foreste remote e incontaminate. I cacciatori possono così approfittare non solo di nuovi territori di caccia, ma anche delle strutture annesse a quest'industria: una rete stradale più sviluppata e una maggiore scelta e facilità di trasporto della carne sino ai mercati cittadini. Inoltre, la nascita e

l'espansione di villaggi costruiti nelle vicinanze delle concessioni forestali, favoriscono il commercio di selvaggina anche in queste zone.

I cambiamenti socio-economici e ambientali ai quali assistiamo, combinati alla diversità genetica e alla prevalenza di infezione del virus dell'SIV nei primati, ci suggeriscono che l'uomo è esposto ancora più di prima a questo virus. Non solo lo scimpanzé o il gorilla o il cercocebo grigio sono cacciati e consumati dall'uomo, ma anche varie specie di cercopitechi e di colobi per esempio. È quindi estremamente importante continuare la ricerca e la caratterizzazione di nuovi virus e di determinarne la prevalenza di infezione nei primati non-umani, al fine di meglio comprendere quali tra questi virus specie-specifici rappresenti un rischio per la salute della popolazione umana.

Lo scopo di questa tesi era quello di:

(i) determinare l'infezione da SIV e eventualmente misurarne la prevalenza nei differenti gruppi sociali di primati che vivono nella foresta di Taï in Costa d'Avorio, sapendo che la caccia di queste specie è una pratica corrente in questa zona

(ii) determinare l'infezione, la prevalenza e caratterizzare a livello molecolare l'SIV presente nella specie di colobo ferruginoso che vive nella Riserva Naturale di Abuko, in Gambia. Due sottospecie diverse di colobo ferruginoso abitano il Gambia e la Costa d'Avorio: ciò rappresenta un'opportunità di valutare se due sottospecie differenti possano essere infettate o meno da virus geneticamente diversi e conseguentemente di poter meglio comprendere il ruolo giocato dalle barriere geografiche sull'evoluzione del virus SIV

(iii) comparare la struttura molecolare dei virus SIV presenti nel colobo ferruginoso e nel colobo verde, due specie differenti ma strettamente apparentate

(iv) determinare se la sottospecie di scimpanzé vivente nell'Africa dell'ovest possa essere infetta da un virus simile a quello delle scimmie del quale si nutre, sapendo che sino ad ora, solo la sottospecie di scimpanzé dell'Africa centrale e dell'Africa dell'est si sono rivelate infette da SIV.

La maggior parte dei risultati di questa tesi sono stati ottenuti grazie all'analisi di dati raccolti in modo non-invasivo. Ciò significa che l'infezione da SIV è stata determinata grazie alla presenza di anticorpi, o all'isolamento di sequenze virali effettuate a partire da campioni fecali raccolti nelle foreste della Costa d'Avorio e in Gambia.

Per poter discriminare l'appartenenza dei campioni fecali raccolti, e quindi determinare in modo accurato la prevalenza di infezione da SIV nei gruppi di primati selezionati, un genotipaggio parziale dell'ospite è stato effettuato a partire dall'analisi del DNA estratto dalle cellule epiteliali rilasciate dall'intestino. Questi dati genetici e virali sono oggi ottenibili grazie all'avvenuto miglioramento delle tecniche di conservazione, di estrazione del DNA, dell'RNA e degli anticorpi, a partire da materiale fecale.

La scelta del Parco Nazionale di Taï e della Riserva Naturale di Abuko non sono casuali: in entrambi i luoghi, si trovano delle popolazioni di scimmie abituate alla presenza dell'uomo da più di dieci anni sulle quali sono state effettuate delle ricerche comportamentali ed ecologiche. Ciò ha permesso un'osservazione più facile dei gruppi di scimmie selezionate e l'integrazione di dati comportamentali precedentemente raccolti allo studio della prevalenza di infezione da SIV. In effetti, questo virus è trasmesso principalmente per via sessuale, ma anche per via verticale (dalla madre al figlio) o attraverso morsi o la contaminazione di ferite aperte.

È quindi importante considerare il sistema di accoppiamento, le caratteristiche di dispersione degli individui, la taglia dei gruppi, il numero medio di maschi adulti nel gruppo, le associazioni polispecifiche, etc. quali fattori favorenti la trasmissione del virus all'interno di un gruppo o di una specie o attraverso le specie.

In totale, più di 300 campioni fecali sono stati raccolti, tra marzo e luglio del 2004, in due gruppi di colobo ferruginoso (*Piliocolobus badius badius*), in due gruppi di colobo orsino (*Colobus polykomos polykomos*), in tre gruppi di colobo verde (*Procolobus verus*), in tre gruppi di scimmie Diana (*Cercopithecus diana*), in un gruppo di cercopitechi di Campbell (*Cercopithecus campbelli*), in un gruppo di cercopitechi dal naso bianco minore (*Cercopithecus petaurista*) e in un gruppo di cercopitechi dal naso

bianco maggiore (*Cercopithecus nictitans*) al confine occidentale della foresta di Taï, in Costa d'Avorio.

Per identificare i campioni fecali raccolti abbiamo vagliato 16 microsatelliti umani in queste sette specie di scimmie. Tra il 25% e il 37% dei marcatori genetici selezionati hanno amplificato con successo il DNA estratto dai campioni fecali di tutte le specie producendo risultati affidabili (Capitolo 5).

Le analisi serologiche effettuate sui campioni fecali di colobi e di cercopitechi sono risultate negative o non interpretabili.

Inseguito sono state eseguite delle trascrizioni inverse della reazione a catena della polimerasi (RT-PCRs) utilizzando sia marcatori genetici universali che più specifici, focalizzanti le regioni della capside del virus (*gag*), dell'enzima polimerasi (*pol)* e della matrice virale (*env*) del genoma SIV. Nessun risultato positivo è stato riportato nelle specie investigate, eccetto per i colobi ferruginosi.

Tra i 53 individui adulti (dedotti grazie alle analisi genetiche dei microsatellite effettuate sui campioni raccolti) appartenenti a due gruppi vicini, 14 sono risultati positivi all'infezione da SIV (chiamato SIVwrc da "western red colobus"). Ciò corrisponde ad una prevalenza del 26% negli individui analizzati. A parte qualche eccezione, le analisi filogenetiche delle sequenze *pol* e *env* hanno rivelato un basso grado di diversità genetica all'interno di ogni gruppo e in generale, un raggruppamento virale in relazione al gruppo sociale di origine. Tuttavia, è stata riscontrata una più elevata diversità genetica tra un gruppo e l'altro. Dati comportamentali e demografici raccolti precedentemente in queste comunità indicano che i colobi ferruginosi vivono in società promiscue costituite da più individui maschi adulti, dove le femmine lasciano il loro gruppo natale allo stadio di sub-adulto e dove le copulazioni al di fuori del gruppo di adozione o l'immigrazione maschile sono state raramente osservate. I profili filogenetici osservati sembrano riflettere queste caratteristiche comportamentali (Capitolo 6).

I risultati negativi ottenuti nelle altre specie potrebbero essere imputati ad una minore trasmissione del virus in relazione alla struttura sociale e al sistema di accoppiamento di queste specie. Altri fattori, quali la difficoltà di monitoraggio degli individui e la conseguente parziale copertura di raccolta della popolazione adulta, la conservazione sul campo dell'RNA virale e la sua fragilità intrinseca ed infine la relativa

sensibilità e specificità dei test rispettivamente serologici e molecolari potrebbero aver contribuito all'ottenimento di tali risultati (Capitolo 9).

Parallelamente, sono stati analizzati 16 campioni fecali appartenenti a altrettanti colobi ferruginosi (*Piliocolobus badius temminckii*) e due campioni di tessuti prelevati da due carcasse della medesima specie. Questa colletta è stata effettuata nella Riserva Naturale di Abuko in Gambia tra gennaio e febbraio del 2005. Nessun campione fecale di colobo ferruginoso di temmincki è risultato positivo alle analisi RT-PCR. La presenza molecolare dell'SIV è stata però evidenziata in uno dei due campioni di tessuto e le analisi filogenetiche delle sequenze parziali di *pol* e *env* hanno mostrato che il nuovo ceppo SIVwrc-*Pbt* virus è strettamente apparentato al ceppo SIVwrc-*Pbb* proveniente dall'ospite *P.b.badius* isolato nella foresta di Taï in Costa d'Avorio. Questo ci suggerisce che sottospecie di colobi geograficamente separate sono infettate con virus strettamente apparentati (Capitolo 7). La caratterizzazione molecolare e le analisi filogenetiche della sequenza genomica virale completa dell'SIVwrc-*Pbt* e di altri due genomi di SIVwrc-*Pbb* susseguentemente sequenziati (Capitolo 8) hanno confermato che l'SIVwrc-*Pbt* e l'SIVwrc-*Pbb* fanno parte di un lignaggio di SIV specie-specifico, sebbene siano apparentati con il lignaggio dei cercopitechi barbuto (SIVlho), dei cercoipthechi dalla coda dorata (SIVsun) e dei mandrilli (SIVmnd-1) lungo l'insieme del genoma virale.

Inoltre abbiamo più recentemente caratterizzato il genoma completo dell'SIV presente nel colobo verde (SIVolc), grazie all'analisi di un campione di sangue prelevato nel corso di uno studio anteriore effettuato anch'esso nel Parco Nazionale di Taï. Questa specie di colobo è apparentata a quella del colobo ferruginoso e le analisi dei loro rispettivi virus confermano l'esistenza di lignaggi differenti ma comunque apparentati lungo l'intero genoma virale (Capitolo 8).

Questi risultati confermano la complessa storia evolutiva dei lentivirus di primate, la quale può essere stata influenzata per lungo tempo dalla co-speciazione tra ospite e virus, ma anche da eventi di trasmissione e ricombinazione virale tra specie diverse. Vi è il bisogno di caratterizzare ulteriori virus in altre specie di colobi per meglio comprendere la relazione filogenetica che lega i loro virus a quelli presenti nei primati

infetti da SIV del lignaggio SIVlho per stabilire se vi è stata ricombinazione tra i loro virus ancestrali.

Infine, 5 campioni di sangue e di tessuti prelevati durante uno studio precedente su altrettante carcasse di scimpanzé del Parco Nazionale di Taï sono stati analizzati per la presenza di SIV. Sino ad ora questo virus non è stato mai isolato nella sottospecie di scimpanzé (*Pan troglodytes verus*) vivente in Africa dell'Ovest. Lo scimpanzé, in generale, caccia specie di scimmie più piccole ed è stato provato che l'SIVcpz che infetta lo scimpanzé dell'Africa centro-occidentale (*Pan troglodytes troglodytes*) è il risultato della ricombinazione virale di SIVs presenti nelle specie di primate di cui si nutre. Secondo questa logica, abbiamo testato i 5 campioni a disposizione utilizzando sia marcatori virali universali sia marcatori più specifici come quelli che amplificano le sequenze virali del SIV presente nel colobo ferruginoso, la preda favorita degli scimpanzé del Parco Nazionale di Taï. Sebbene le analisi serologiche evidenziassero una positività leggera in 3 di questi campioni, non abbiamo potuto confermare questi risultati tramite le analisi PCR. Questo risultato sembrerebbe dimostrare che questa sottospecie sia realmente risparmiata da questo virus, oppure che sia infetta da un SIV talmente divergente che gli strumenti molecolari a disposizione non sono in grado di evidenziare (Capitolo 10).

Complessivamente, considerando la prevalenza di infezione da SIVwrc in natura, la relativa abbondanza di colobi ferruginosi, la loro coabitazione con altre specie di primati potenzialmente portatrici di SIV geneticamente diversi, considerate la caccia e il consumo di carne di colobi ferruginosi da parte e dello scimpanzé e da parte dell'uomo, vi sono i prerequisiti per un'eventuale trasmissione virale da primate a primate o da primate all'uomo. La pandemia di HIV-1 odierna è il risultato di poche trasmissioni da primate all'uomo. Un numero maggiore di trasmissioni potrebbe prodursi o essersi già prodotto ma non essere stato ancora evidenziato sia per ragioni pertinenti alla sensibilità e alla specificità delle tecniche serologiche e molecolari a disposizione o semplicemente per ragioni di isolamento geografico delle popolazione umane a rischio. Oltre alla necessità di sviluppare test serologici più sensibili e strumenti molecolari più specifici, sarebbe opportuno valutare la capacità dei ceppi virali recentemente isolati ad infettare cellule umane, misurandone le capacità di crescita e il potenziale citotossico.

Questi risultati dovrebbero aiutarci a valutare in modo critico le circostanze e i fattori che governano le trasmissioni di SIV attraverso le specie e dunque permetterci di meglio comprendere il reale rischio di infezione per la popolazione umana. Per di più, visto l'aumento della prevalenza di HIV nelle zone rurali d'Africa, la potenziale ricombinazione di SIVs con i differenti ceppi di HIV circolanti potrebbe rappresentare un ulteriore fattore di emergenza di un nuovo tipo di HIV.

# Outline of the thesis

Chapter 1 Gives a general introduction to the origin of human immunodeficiency virus (HIV), the molecular characteristics of simian immunodeficiency virus (SIV) and its genetic features in relation to host diversity and evolution. It provides also an overview of the issue of bushmeat hunting and consumption of non-human primates in West Africa in relation to the potential of SIV cross-species transmission and the risk of emergence of new zoonoses

Chapter 2 States the aims and objectives of the PhD thesis

Chapter 3 Gives an overview of the PhD history and collaborations

Chapter 4 Describes the study areas, the primate species investigated and the methods used

Chapter 5 Presents the results of microsatellite analysis on 7 monkey species from the Taï Forest, Côte d'Ivoire

Chapter 6 Presents the results on the prevalence and genetic diversity of SIV infection in wild-living western red colobus monkeys from the Taï Forest, Côte d'Ivoire

Chapter 7 Presents the results on the molecular characterisation of a complete genome of SIV isolated from a subspecies of western red colobus from Abuko Nature Reserve, The Gambia

Chapter 8 Presents the results on the molecular characterisation of three complete genomes of SIV isolated from two western red colobus and one olive colobus from the Taï Forest, Côte d'Ivoire

Chapter 9 Presents the results and the limitations of non-invasive research on SIV infection among several wild-living primate species from the Taï Forest, Côte d'Ivoire and discusses the possible influence of demographic, ecological and behavioural data on the results obtained

Chapter 10 Presents the results on SIV analysis of 5 blood samples from western chimpanzees from the Taï Forest, Côte d'Ivoire

Chapter 11 Gives a general discussion of the methodology and of the main results

Chapter 12 Provides conclusions and perspectives

*Curriculum vitae*

# 1. Introduction

## Foreword

Recent decades have seen the appearance of many new infectious diseases in human populations, including Severe Acute Respiratory Syndrome, Ebola hemorrhagic fever, hantavirus pulmonary syndrome and HIV/AIDS [121, 126, 246, 329]. The factors that underline their emergence remain sometimes elusive. Most of the human emerging infectious diseases known are of viral origin. Several analysis found that from 61% up to 90% of human pathogens are “shared” with animal hosts [10, 369]. As humans and wildlife continue to get in close contact for different reasons, it is important today to better understand the factors that influence disease occurrence in wildlife and livestock in order to be able to better predict disease emergence on a global human scale. Non-human primates (NHP) represent a reservoir of micro and macro parasites for humans who, for different reasons, get in close contact with them. Moreover, our close phylogenetic relationship represents an additional factor favouring pathogens transmission between human and non-human primates [379]. NHP are probable reservoirs for a variety of helminth infections in Africa, including multiple species of *Schistosoma* [255], *Strongyloides fulleborni* [314], genera such as *Brugia*, *Dirofilaria*, *Taenia*, and *Trichiuris*, and protozoa such as *Entamoeba*, *Giardia*, *Leishmania* and *Trypanosoma* [264]. Several malaria species show evidence of cross-species transmission between humans and wild primates [265, 286]. Even so, over half of the “shared pathogens” listed as emerging in humans are viruses, including several vector-borne pathogens. A large number of viruses have been isolated from wild primates. Some more commonly known DNA viruses include those in the families Poxviridae (monkeypox virus), herpesviridae (*Simplexvirus* and *Varicellovirus*) and Papovaviridae (*Papillomavirus*). Among humans and other primates, RNA viruses include those from the families Flaviridae (yellow fever and dengue fever virus), Ortho- and Paramyxoviridae (influenza and measles viruses), and Retroviridae (simian immunodeficiency virus, simian-T-lymphotrophic virus, foamy virus) [265, 286].

A range of activities involves direct contact between humans and NHPs and allow for the transmission of micro-organisms. Such behaviour can facilitate transmission of micro-organisms from NHPs to humans [411], with consequences for human health, as well as from humans to NHPs, with consequences for wildlife conservation [398]. Care for captive nonhuman primates has lead to the transmission of a range of infections, including simian foamy virus [148], herpesvirus B (HBV) [166], primate malaria [69], and tuberculosis [179]. Nonhuman primate ecotourism (e.g., gorilla watching) has been associated with the possible transmission from humans to NHPs of diseases that include scabies (*Sarcoptes scabiei*) [137], intestinal parasites [348], and measles [49]. Exposure to sick or dead primates represents a risk of infection not only for the local population, but also for researchers conducting fieldwork, as in the case of a student who was infected by an Ebola virus transmitted while performing an autopsy of a chimpanzee carcass in Taï National Park, Côte d'Ivoire [108]. Laboratory handling of tissues or fluids of NHPs has lead to transmission of a range of infections to humans, including simian immunodeficiency virus (SIV) [184] and SV40, which was subsequently distributed through oral polio vaccine to millions of people [330]. Additionally, keeping NHP pets has been linked to transmission of a variety of micro-organisms [311]. Finally, hunting and butchering NHPs have been linked to the transmission of Ebola, monkeypox [415], and simian foamy virus [413]. Because of the broad range of fluid and tissue types involved with hunting and butchering, this mechanism of transmission may be particularly important in cross-species transmission. A number of important human diseases, including adult T-cell leukaemia (HTLV-1) and malaria (*Plasmodium* spp.), are believed to have emerged as the results of ancient or contemporary cross-species transmissions from NHPs [347, 412]. However, the most striking and devastating example of emerging disease resulting from cross-species transmission from non-human primates is that of HIV/AIDS.

An additional source of concern is that transmission of SIV associated with hunting and butchering of NHPs could be an ongoing process and that contemporary hunters may yet be found with SIV infections. This hypothesis is strengthened by recent evidence suggesting that hunted NHPs are infected with SIV at a high rate [287]. Another retrovirus, named simian foamy virus (SFV) is ubiquitous in NHPs and its

transmission to humans has been also associated with hunting practices [413]. However, foamy viruses are unique among the retroviruses in having no disease association (for a review on SFV, please refer to Murray and Linial, 2006). Moreover, in many areas of the world, but mainly in Africa, hunting has shifted from a primarily subsistence activity to an organized, commercial venture. Factors underlying the increase of bushmeat demand include human population growth, the absence of effective alternatives to hunting for meat, appealing short-term economic benefits from the commercial bushmeat trade, lack of capacity to enforce national and international legislation, and logging [45]. Given these circumstances, it is essential to survey further potentially successful cross-species transmissions by continuing to characterise the presence and the prevalence of SIV in different species of primates in order to prevent the spread of novel, emerging HIV infections.

In the introductory chapter of this thesis I am going first of all to give an overview of the human immunodeficiency virus responsible for the acquired immunodeficiency syndrome in humans. I will briefly portray its genomic organisation, viral structure and replication cycle. Subsequently, I will describe the patterns of HIV transmission and its evolution to AIDS starting from the primary HIV infection and terminating by briefly mentioning the diagnostic methods available today. Finally, I will illustrate the HIV genetic diversity, its classification, geographic distribution and epidemiology. The origin of HIV will then be addressed as a starting point for examining the role of NHP as a continuing source for human emerging diseases. The prevalence and molecular characteristics of simian immunodeficiency virus in the wild and its patterns of transmission among the NHP hosts are the main topic investigated in this thesis. Therefore, the second part of this introduction will focus on SIV genetic diversity and evolution as well as on epidemiology and routes of transmission of the virus. The general lack of SIV pathogenecity in its natural host will be also addressed. An important part of this study is related to the behavioural and ecological characteristics of the host, which play a critical role in the spread of a virus across individuals or species. Hence, the acquired knowledge on demographics, feeding ecology, social structure, mating system and patterns of dispersal of the wild-living primate species will be discussed.

Finally, I will provide a description of the diagnostic and non-invasive methods of SIV detection and host identification that better mirror the situation in the wild. These techniques will then be explored in greater details in the “Materials and methods” section.

## 1.1 Human Immunodeficiency Virus (HIV)

### 1.1.1 Taxonomic classification of lentiviruses

HIV and SIV belong to the *Lentivirus* genus (from the Latin *lentus* for “slow”) of the *Retroviridae* family. A taxonomy based on the phylogenetic evolution classifies the retroviruses in 7 genera: *Lentiviruses*, *Alpharetroviruses*, *Betaretroviruses*, *Deltaretroviruses*, *Gammaretroviruses, Epsilonretroviruses* and *Spumaretroviruses* (Figure 1.1). Retroviruses are so called because their RNA genome is transcribed into DNA within the host cell using the viral enzyme reverse transcriptase (RT) and integrates into the genomic DNA of the host cell as a provirus [70]. Lentiviruses are exogenous (transmitted horizontally form host to host) and are only distantly related to endogenous germ line retroviruses [129]. They include viruses infecting primates, felids and a variety of wild and domesticated ungulates and are known to be associated with a long incubation period. In non-human primates (NHPs) these infections appear to be clinically silent. Infection in certain felid species (i.e. the domestic cat) results in immunodeficiency. Ovine and caprine lentiviral infections may result in neurological disorders, arthritis, and pneumonia, while equine lentiviral infections result in recurrent fever and blood dyscrasias [213, 241].

Other retroviruses infecting human and non-human primates are HTLV (Human T-Cell Leukaemia Virus) and STLV (Simian T-Cell Leukaemia Virus). These viruses belong to the *Deltaretroviruses*, which, together with the *Alpha-, Beta-, Gamma-* and *Epsilonretroviruses (Oncovirinae*) induce T-cell leukaemia and are associated with neurological disorders such as the Tropical spastic paresis [213].

Finally, foamy viruses (FV), also known as spumaviruses, are the only genus of the Spumaretrovirinae subfamily of retroviruses. FV have been found in NHPs, cats, cows, and horses as well as in humans who have acquired the infection from NHPs.

In all hosts, FV establishes a lifelong, persistent infection with no associated pathologies [251].

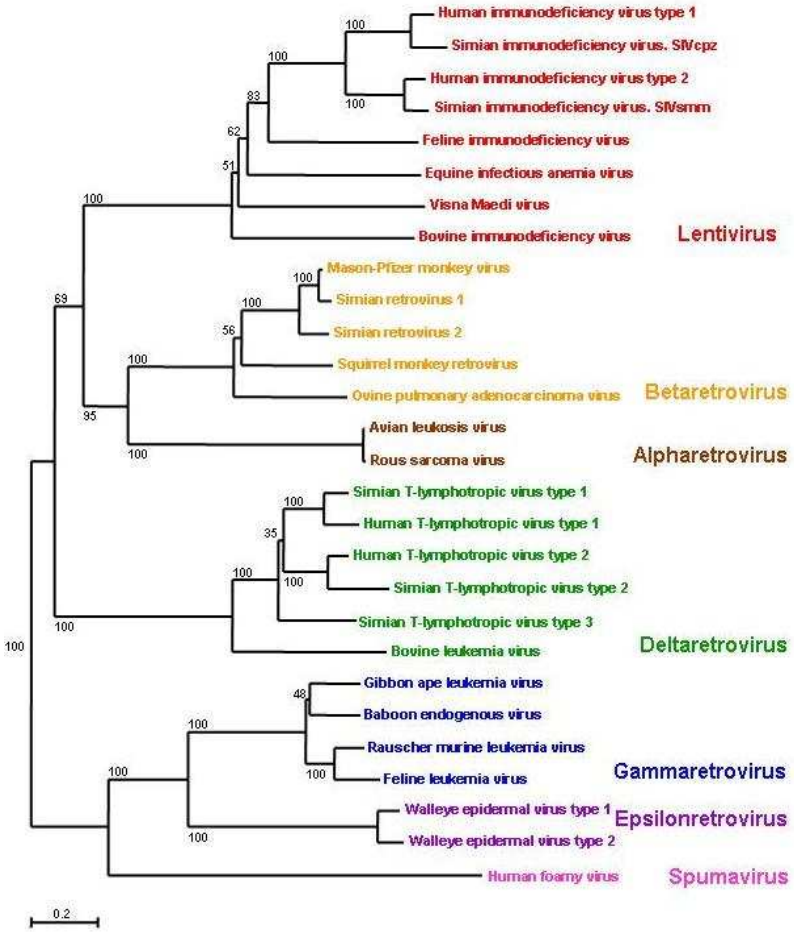



Figure 1.1: Taxonomic classification of *Retroviridae* in seven genera.The phylogenetic tree was inferred by the neighbour-joining method [316] and based on 28 Pol (RT-integrase) sequences of 670 amino acids extracted from the Genbank (Courtesy of A. Aghokeng)

### 1.1.2 HIV genomic organisation and viral structure

The genomic size of HIV is about 10 kilo bases (kb), with open reading frames coding for several proteins. The virion is about 100 to 120 nm in diameter [199]. All primate lentiviruses have a common genomic structure, consisting of the long terminal repeats (LTR), which flank both ends of the genome and are essential for viral integration and transcription, three structural genes, *gag*, *pol* and *env* and five accessory

genes, *vif, vpr, tat, rev* and *nef*. Some primate lentiviruses carry an additional accessory gene, *vpx* (e.g. HIV-2) or *vpu* (e.g. HIV-1) in the region between *pol* and *env*.

The structural genes *gag*, *pol* and *env* code respectively for the core proteins (p17, p24 and p7), the viral enzymes integrase, protease and reverse transcriptase, and the glycoproteins of the envelope (gp120 and gp41) (Figure1. 2).

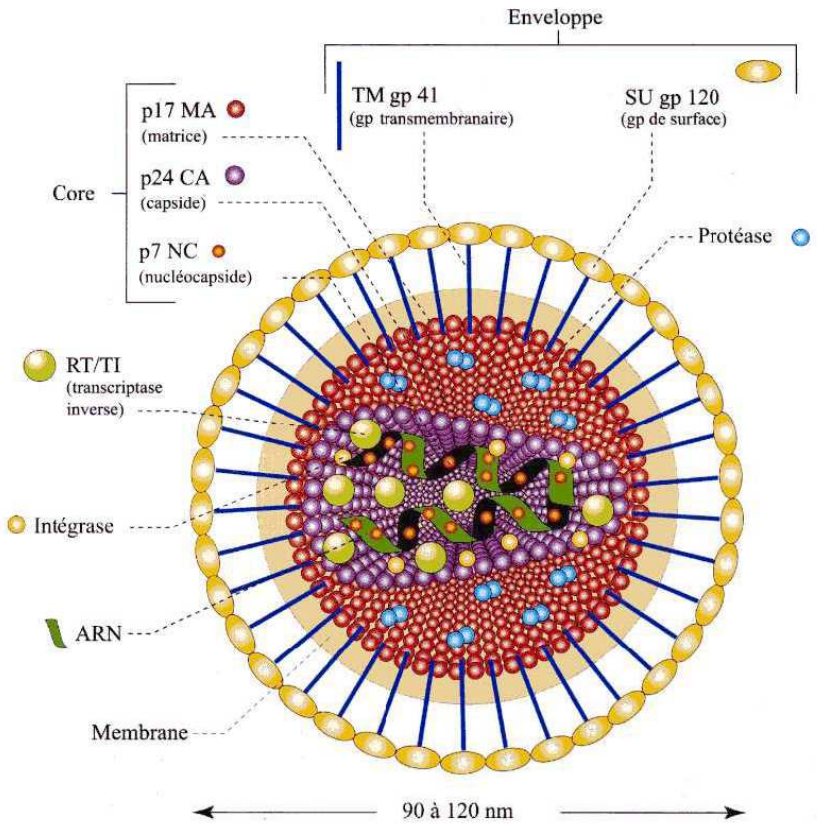



Figure 1.2: Structure of an HIV virion. Source:Traité de Virologie Médicale. J.M. Huraux, éditions Estem, 2003

### 1.1.3 HIV replication cycle

The infection begins when a HI-Virus encounters a host cell expressing a receptor on its surface called cluster designation 4 (CD4) [203, 267] (Figure 1.3). After the attachment to the CD4 molecule, the gp120 protein of the envelope is displaced, leading to the uncovering of the variable domains V1/V2 on the envelope gp41, which

exposes the V3 loop to a proteolitic enzymatic activity necessary for virus-cell fusion [245, 324]. The presence of additional receptors is needed for virus- cell fusion: the CCR5 coreceptor present on macrophage tropic isolates [65] or the CXCR4 coreceptor for the T cell line tropic strains [25].Two types of viruses can be therefore distinguished: R5 and X4 viruses respectively [24].

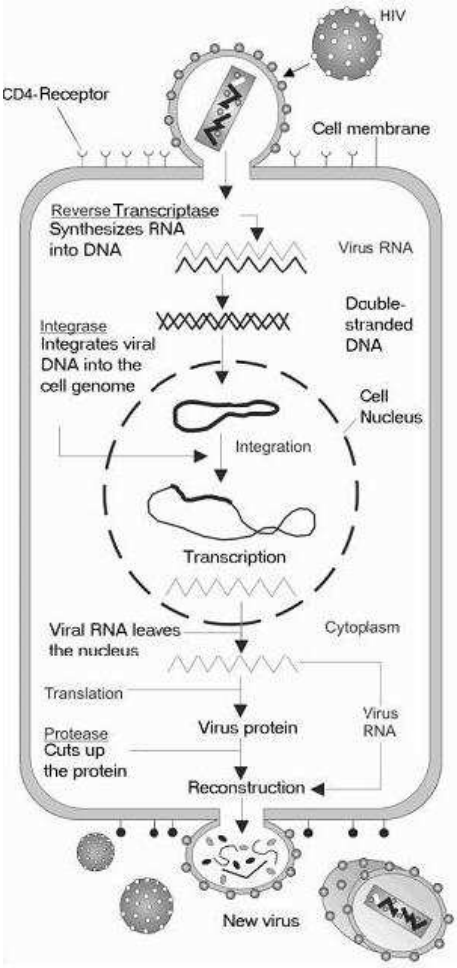



Figure 1.3: Replication cycle of HIV. Source: http://de.wikipedia.org/wiki/Bild:Hiv_gross.png)

Once the virus has entered the host cell, the HIV RNA exits from the viral capsid with the help of the capsid protein p24 [224]. Still associated with core proteins, the viral RNA undergoes reverse transcription by using its RNA- and DNA-dependent DNA polymerase and RNase H activities and eventually forms a double stranded DNA copy of its genome (cDNA) [44, 213]. This cDNA is transported to the nucleus and then becomes integrated into the cell chromosome via a process involving integrase and host

cell factors (for reviews, see[80]). Following virus integration, the earliest mRNA species made in the infected cell are doubly spliced transcripts encoded by the major regulatory genes, particularly *tat*, *rev*, and *nef*. mRNA transcription relies primarily on binding to the Long Terminal Repeat by cellular transcription factors such as nuclear factor Kappa B (NK-κB), NFAT, AP-1, SP-1 and the Tat binding proteins [138, 213].

Viral assembly takes place at the cell membrane, where viral RNA is incorporated into capsids that bud from the cell surface, taking up the viral envelope protein. The Env polyprotein (gp160) is transported through the endoplasmatic reticulum to the Golgi complex where it is cleaved by a cellular protease into the two envelope glycoproteins gp41 and gp120 and subsequently glycosylated by other cellular enzymes. Cleavage of the Gag-Pol polyprotein either occurs in the forming bud or in the immature virion after it buds from the host cell. During this maturation, the viral protease splits the polyproteins into the individual HIV core proteins p14, p24 and p7 and into the three enzymes protease, reverse transcriptase and integrase. The assembly of the viral proteins with the two genomic RNA molecules at the level of the plasma membrane of the host cell is facilitated by the Vpu and Vif proteins. A mature HIV virion is then extruded in the extracellular environment by a mechanism of budding and is then able to infect another cell [258].

### 1.1.4 HIV transmission

HIV can be transmitted horizontally, via blood or sexual contact, and vertically, from the mother to the child. Blood transmission primarily affects intravenous drug users, sharing contaminated needles or syringes, hemophiliacs, and other patients who receive a transfusion of blood or a product derived from blood. Sexual transmission of the HIV infection occurs through the mucosal tissue of the vagina, vulva, penis and rectum, rarely via the mouth or the upper gastrointestinal tract. More than 80% of the HIV infections in the world are attributed to unprotected sexual intercourse [380]. Transmission of virus from mother to child can involve direct infection of the fetus in utero, or during birth, by exposure of the newborn to maternal blood and secretions. Maternal milk is also a source of infectious HIV that can be transferred to the newborn [210].

### 1.1.5 From primary HIV infection to evolution to AIDS

The HIV infection and its progression to AIDS is divided mainly in three stages.

The first stage immediately follows the individual's exposure to HIV and it is characterised by a high viral replication possibly reaching $10^6$- $10^7$ copies/ml in the plasma and by a significant drop of circulating CD4+ T cells. Two to four weeks after exposure to the virus, virtually all patients suffer from flu-like symptoms. The immune system stimulates the activation of killer T cells (CD8+ T cells), which destroys HIV-infected cells and generates B cell-derived antibodies, resulting in seroconversion. The CD8+ T cell response is important in controlling virus levels and CD4+ T cell count may rebound to around 800 cells/ml (normal values around 1200 cells/ml). A good CD8+ T cell response may slow disease progression, although it does not eliminate the virus [9].

The second stage is represented by the clinical latency or asymptomatic stage of infection and can vary between a few months up to more than 15 years. At this stage the virus can lay dormant within the chromosomes of the infected cells and constitutes an inactive reservoir of provirus in the lymphoid organs.

The third stage is reached when the number of CD4+ T cells declines below a critical level. The host immunity is compromised and the patient progresses to AIDS. Non-specific signs and symptoms as fatigue, weight loss, recurring respiratory tract infections and oral ulcerations appear but the main concerns are opportunistic pulmonary infections (*Pneumocystis carinii*), cytomegalovirus infections, fungal (*Candida albicans*) and mycobacterial (*Mycobacterium tuberculosis*) infections and malignant neoplasms such as Kaposi's sarcoma and lymphomas [213].

### 1.1.6 Diagnosis of HIV infection

The diagnosis of HIV infection is established primarily by the detection of viral antibodies. However, there is an initial serological window of about three weeks when HIV infection can be diagnosed only by detecting viral RNA, or viral p24 antigen.

Viral RNA can be found in the blood already a few days after exposure to the virus, which can reach levels of more than $10^6$ copies/ml. The p24 antigen is detected between 15 days and 30 days after infection and it is usually preferred as a measure in

suspected mother-child HIV transmissions, where the antibodies detected could be those of the mother.

Around three weeks after infection, the body starts to produce HIV antibodies, which can be detected by the Enzyme-Linked ImmunoSorbent Assay (ELISA) or Enzyme ImmunoAssay (EIA), which is based on the principle of specific antibody-antigen binding. The antigens used are generally recombinant proteins or synthetic peptides representing as much as possible the HIV genetic diversity.

The detection of p24 antigen and HIV antibodies are combined in the 'fourth-generation tests' reducing the diagnostic window by 2-7 days. However, given the high sensitivity of the ELISA tests, there is a slight risk of false positive results; therefore, ELISA positive results need to be confirmed by an additional, more specific test, such as western Blot or Line Immuno Assay (LIA).

There are also rapid tests that are particularly suitable in situations where resources, equipment and time are scarce. Serum, plasma, whole blood or saliva can be tested. They have similar accuracy rates as traditional ELISA/EIA screening tests, although their sensitivity is less compared to the fourth generation tests (in terms of detecting very early infections cases). They are based on agglutination or adsorption of the antigen-antibody complex to a membrane, followed by a colour development visible to the naked eyes [214].

### 1.1.7 Classification and geographic distribution of HIV

AIDS is caused by two types of lentiviruses: HIV-1 and HIV-2. Whereas HIV-1 has spread globally, HIV-2 has remained largely restricted to West Africa. Lower viral loads in HIV-2 infected patients are thought to be correlated with a lower transmissibility and a longer clinic latency, which may explain the difference in the global epidemiology between HIV-1 and HIV-2 [331].

Based on phylogenetic analyses of numerous isolates obtained from diverse geographic origin, HIV-1 is classified into three groups: M, N, and O. Group M (for major) represents the vast majority of HIV-1 strains. It is found worldwide and is responsible for the pandemic. Groups O (for outlier) and N (non-M, non-O) remain restricted to West-central Africa [60, 345]. Within group M, most sequences fall within a

certain number of discrete clades, allowing the classification of HIV-1 M strains into nine subtypes (A-D, F-H, J, and K), circulating recombinant forms (CRF01-CRF32), and unique recombinants.

The geographic distribution of the different HIV-1 M variants is very heterogeneous (Figure 1.4), and specific distribution of the various subtypes are seen among the different continents, even from country to country or within countries. In Africa, all groups, and many circulating recombinant forms have been documented [147, 234].

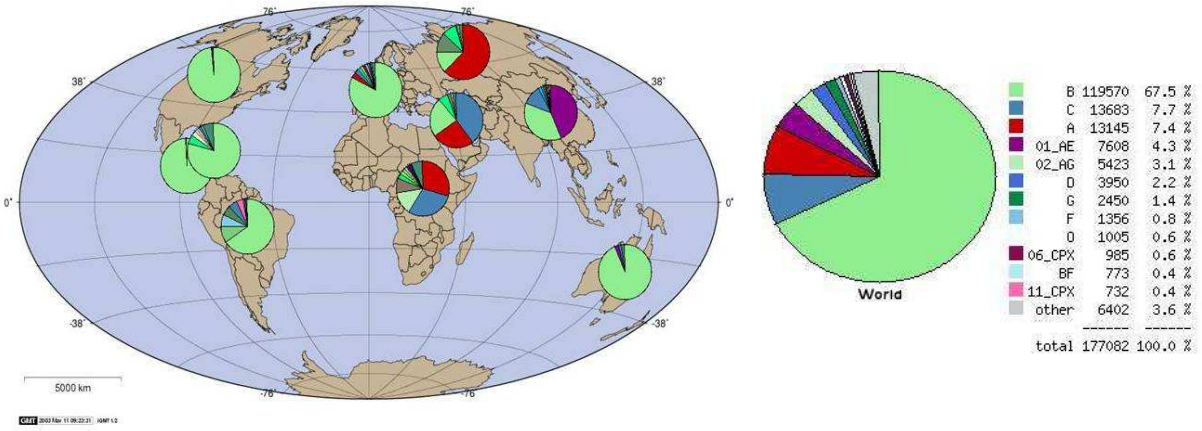



Figure 1.4: Subtypes and CRF distribution in the world based on the available HIV-1 sequences in the Los Alamos HIV-1 database (source: http/www.hiv/lanl.gov/ March 2008)

Compared to HIV-1, only a limited number of HIV-2 strains have been genetically characterised, and eight groups (A-H) have been reported. The geographical distribution of HIV-2 is less extensive than that of HIV-1 (Figure 1.5). Numerous reports indicate that the virus originated in West Africa, where it is endemic and subsequently spread to other more distant African countries and out of Africa into Europe, America and Asia. In West Africa, groups A and B are predominant, whereas all other groups (C-H) have been described for unique individuals [123, 418]. Group A is endemic along the Atlantic coast of West Africa (Senegal, Guinea Bissau, Cape Verde) and group B predominates in the countries of the Gulf of Benin, in particular in Côte d'Ivoire, Mali and Burkina Faso [212, 296, 309]. In Europe the HIV-2 prevalence is low, except for Portugal and France.

In Asia, the HIV-2 distribution is almost exclusively restricted to India and to a few cases in Japan [212].

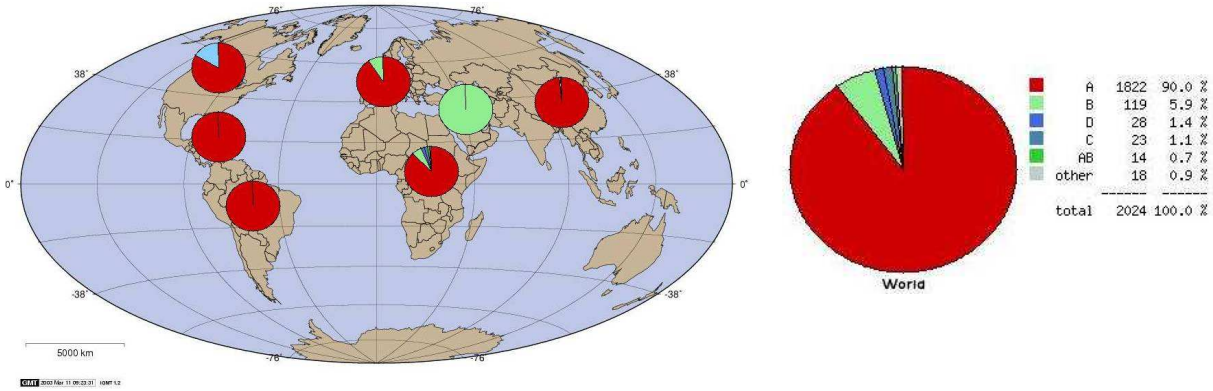



Figure 1.5: World genetic distribution of HIV-2 based on the available HIV-2 sequences in the Los Alamos HIV-1 database (source: http/www.hiv/lanl.gov/ March 2008)

### 1.1.8 HIV genetic diversity

Genetic variation is inherent to all RNA viruses, but has been best characterised for HIV-1. The extensive heterogeneity observed in the worldwide epidemic originates from the rapid viral turnover ($10^{10}$ viral particles/day) in an HIV-infected individual, high rate of incorrect nucleotide substitutions during HIV reverse transcription ($10^{-4}$/nt) in the absence of proof-reading mechanisms, and the pliant conformations/functions of many HIV-1 proteins [156, 401]. In addition to this rapid accumulation of minor genotypic changes, different HIV-1 strains can also recombine at a high rate (estimated at one to three times per viral replication cycle), generating a growing number of recombinant viruses and circulating recombinant forms (CRF) [43, 163, 371, 397]. These CRFs are driven by super- and dual infections, particularly occurring in regions where multiple sub-types co-circulate [157, 293]. Recombination is observed not only between different subtypes, but also between different groups of HIV. Inter-group recombination has been described in West-central Africa between group M and O [292, 365]. HIV-1, like other RNA viruses, has a high mutation rate ($3 \times 10^{-5}$ mutations per base pair per cycle) [227], which, coupled with selection and rapid turnover, results in the generation of swarms of mutants known as viral quasispecies [401]. These quasispecies are subjected to

selection and evolution. Viral mutants' production results in adaptation to environmental changes like host immune system positive selective pressure and antiretroviral therapy.

### 1.1.9 AIDS epidemic

In 2007, the estimated number of persons living with HIV worldwide was 33.2 million (30.6–36.1 million) and the number of deaths due to AIDS in that year was 2.1 million (1.9–2.4 million) (Figure 1.6). AIDS remains a leading cause of mortality worldwide and the primary cause of death in sub-Saharan Africa. More than two out of three (68%) adults and nearly 90% of children infected with HIV live in sub-Saharan Africa, and more than three in four (76%) AIDS deaths in 2007 occurred there, illustrating the unmet need for antiretroviral treatment in Africa. It is estimated that 1.7 million (1.4 million–2.4 million) people were newly infected with HIV in 2007, bringing to 22.5 million (20.9 million–24.3 million) the total number of people living with the virus in the African continent. Unlike other regions, the majority of people living with HIV in sub-Saharan Africa (61%) are women. The region's epidemics, however, vary significantly in scale, with national adult (15–49 years) HIV prevalence ranging from less than 2% in some countries of the Sahel to above 15% in most of southern Africa [380].

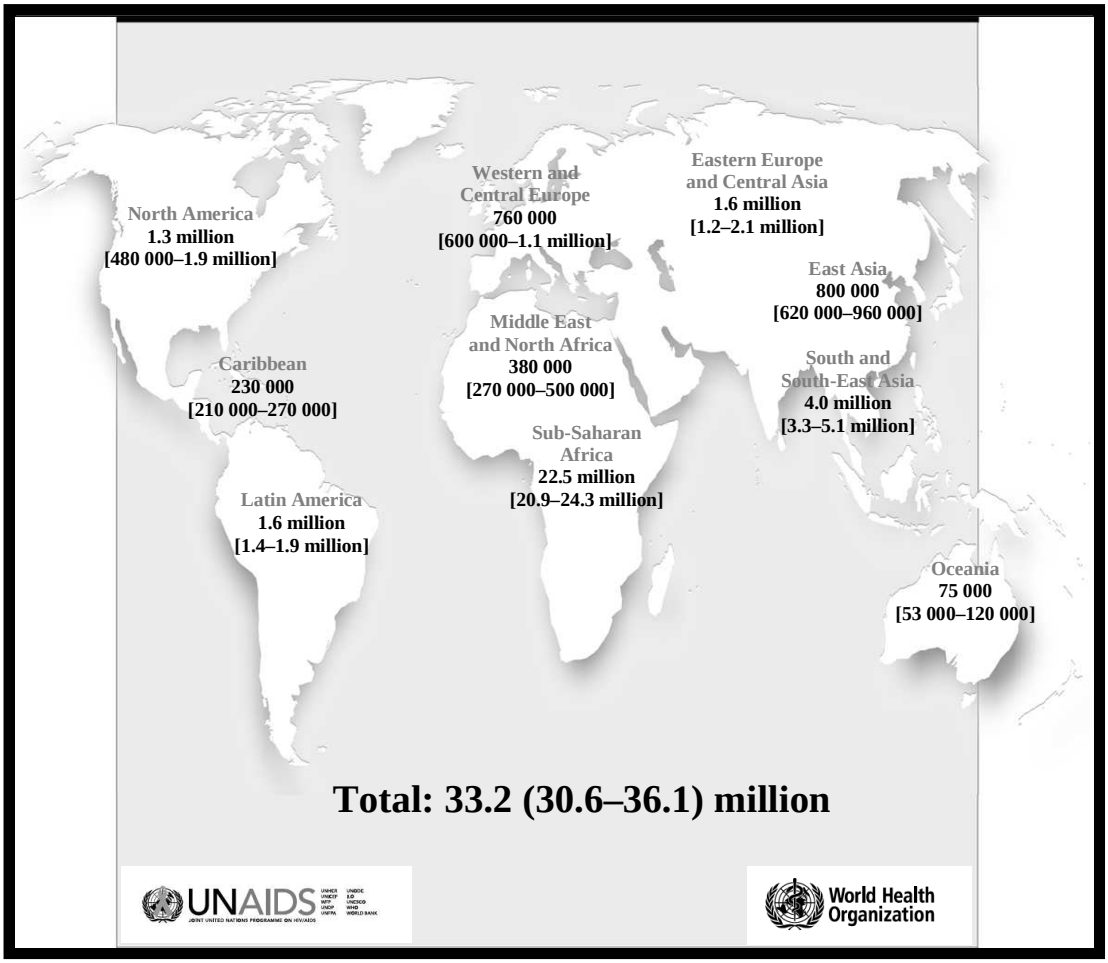



Figure 1.6: The estimated number of persons living with HIV worldwide in 2007. Adapted from [380]

## 1.2 The origin of HIV-AIDS

Twelve years before the emergence of AIDS and again in the mid 1970s two outbreaks of lymphomas adenopathy occurred in a Californian primate research centre in captive rhesus macaques (*Macaca mulatta*) and stump-tailed macaques (*Macaca arctoides*) respectively [222, 226, 359]. At that time, these two epizootics were not recognised as having an infectious origin, even though immune suppression and opportunistic infections were found. These macaques had been in contact with healthy sooty mangabeys before the occurrence of these outbreaks. Retrospective analysis showed that these sooty mangabeys were SIV positive and were recognised as the possible source of SIV in macaques (SIVmac) [226, 249]. At that time, HIV-1 had already been identified as the cause of AIDS [18], but the identity of its natural host was still a mystery. HIV-1 was related to SIVmac, but did not share all the structural features with the latter virus [56]. Moreover, macaques could not be the natural reservoir of SIVmac, since (i) wild-caught species of Asian macaques failed to reveal any evidence of SIV and (ii) macaques were even more susceptible to the disease than humans [417]. In 1986, HIV-2 was isolated [66]. This virus was shown to be closely related to the SIVsmm [62]. Within a very short period of time, significant evidence of lentivirus circulation in African monkeys and apes pointed to a simian origin of AIDS [155, 290].

### 1.2.1 SIVsmm and the origin of HIV-2

Molecular analyses revealed that HIV-2 and SIVsmm were closely related to each other and to SIVs from macaques [152]. The isolation and characterization of SIVsmm strains from captive [7, 110, 152, 217], free-ranging [7, 62] and pet [232] sooty mangabeys in their natural habitat in West Africa (from the Casamance river in Senegal to the Sassandra/N'zo rivers in Côte d'Ivoire) [187] clearly confirmed that mangabeys are the natural host for SIVsmm [62, 63, 291].

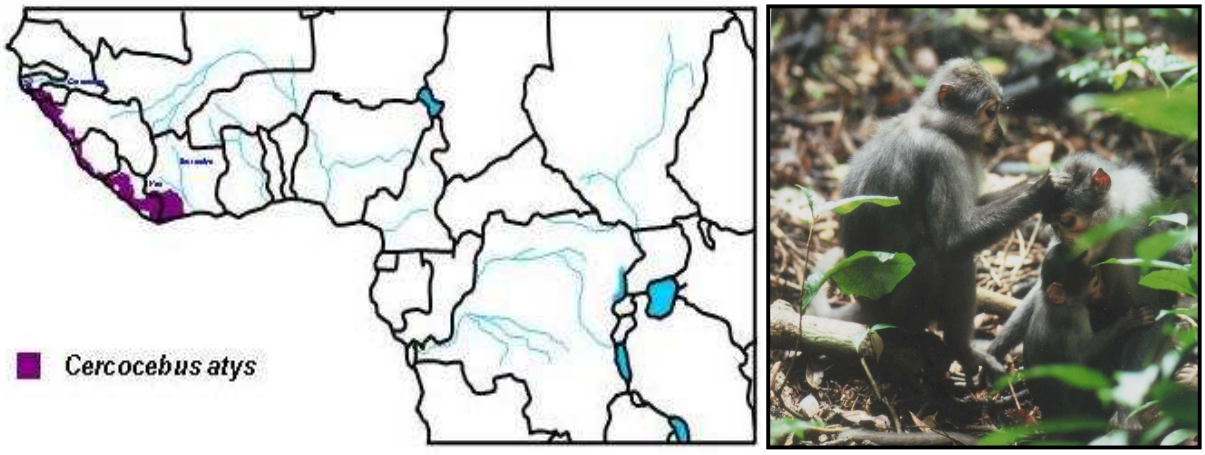



Figure 1.7: Geographic distribution of sooty mangabeys (*Cercocebus atys*) in West Africa.
Adapted from [187]
Picture 1.1: Grooming session among sooty mangabeys in Taï National Park – S. Locatelli

SIVsmm prevalence can reach up to 50% in adult animals in the wild [7, 322]. The natural habitat of sooty mangabeys coincides with the geographical region where HIV-2 is prevalent in West Africa (Figure 1.7). Sooty mangabeys are regularly hunted for food or kept as pets, allowing thus direct contact with humans [232]. Several of the eight HIV-2 subtypes (A-H) have only been found in countries where sooty mangabeys are present [82, 418]. Only subtypes A and B are largely represented in the HIV-2 epidemic, with subtype A being predominant in the western part of West Africa (Senegal, Guinea-Bissau) and subtype B in Côte d'Ivoire [81, 102, 296]. The other subtypes have been documented in one or few individuals only. Except for subtype G, which was isolated from a blood donor in Côte d'Ivoire [418], and subtype H also originating from a patient from the same country [82], subtypes C, D, E and F were isolated in rural areas in Sierra Leone and Liberia and these viruses are more closely related to the SIVsmm strains obtained from sooty mangabeys found in the same area than to any other HIV-2 strains [62, 65]. HIV-2 and SIVsmm lineages are phylogenetically interspersed (Figure 1.8). This suggests that the different clades of HIV-2 must be the result of multiple independent cross-species transmissions of SIVsmm into the human population [143]. In a recent study, evolutionary tree analyses revealed significant clustering of SIVsmm strains from Taï National Park, with five of the eight recognized groups of HIV-2,

including the epidemic groups A and B, thus pointing to a likely geographic origin of these human infections in the eastern part of the sooty mangabeys range [322].

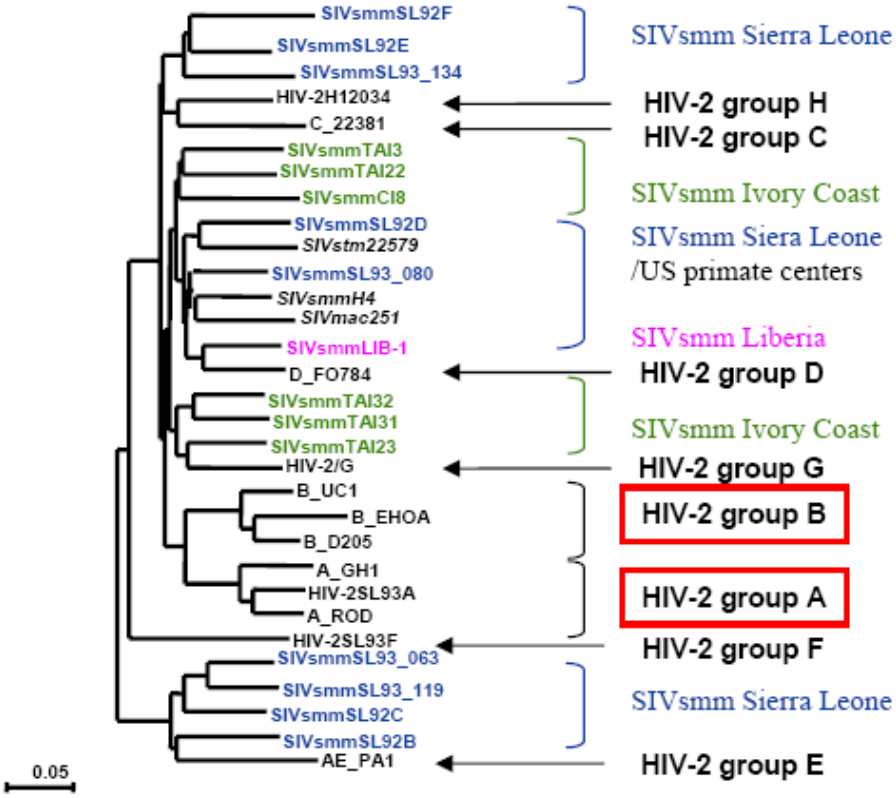



Figure 1.8: Phylogenetic tree illustrating the classification of the different subtypes of HIV-2 in relation to SIVsmm strains, and indicating at least 8 cross-species transmissions of SIVs to humans. The tree has been built by the neighbour joining method [316] (Courtesy of Fran Van Heuverswyn).

### 1.2.2 SIVcpz, SIVgor and the origin of HIV-1

The first SIVcpz strains (SIVcpzGAB1 and SIVcpzGAB2) were isolated from two captive wild-born chimpanzees from Gabon more than 15 years ago. Although these viral strains were closely related to HIV-1, it remained uncertain whether this virus was the immediate ancestor of HIV-1 [165, 290]. In the following years, additional complete or partial SIVcpz genomes were characterised. These include SIVcpzCAM3, SIVcpzCAM5 and SIVcpzCAM13 from chimpanzees from Cameroon, SIVcpzUS from a

chimpanzee (Marylin) kept in a primate centre in the USA and SIVcpzANT from a chimpanzee (Noah) originary from the Democratic Republic of Congo (DRC), illegally introduced into Belgium (Antwerp). Phylogenetic analysis of these SIVcpz strains revealed that SIVcpz is more closely related to HIV-1 than to any other SIV. Furthermore, like HIV-1, all SIVcpz strains harboured the accessory gene *vpu*. Among these chimpanzee viruses, SIVcpzANT showed a high degree of divergence compared to the other SIVcpz strains [288]. Host subspecies identification revealed that the SIVcpzANT strain was isolated from a member of the *P.t.schweinfurthii* subspecies, whereas the other chimpanzees belonged to the *P.t.troglodytes* subspecies. These findings suggested that two distinct SIVcpz lineages were present and that they were congruent with the phylogenetic trees of the host species: SIVcpz*Ptt* and SIVcpz*Pts* from west-central and eastern chimpanzees respectively (Figure 1.9).

A new strain of HIV-1, HIV-1 group N, discovered in 1998 in Cameroon, was even more closely related to SIVcpz*Ptt* [345]. While these data pointed to the West-central African chimpanzees (*P.t.troglodytes)* as the natural reservoir of the ancestors of HIV-1, evidence of SIVcpz*Ptt* infection in wild-living apes was still missing [338]. The low SIVcpz infection rate observed till then raised doubts about the identification of the true virus reservoir. The previously studied chimpanzees were wild-born animals captured at a very young age; therefore their SIV prevalence may not have mirrored the real rate of SIV infection of the wild-living populations. Chimpanzees are an endangered species and may live in very secluded forests. With the advent of non-invasive methods to detect and characterize SIVcpz in faecal and urine specimens, the search for new SIVcpz strains in wild ape populations began to rise. The first full-length SIVcpz sequence (SIVcpzTAN1) obtained from a faecal sample belonged to a wild *P.t.schweinfurthii* chimpanzee from Gombe National Park, Tanzania [321]. Subsequently, additional cases of SIVcpz*Pts* infections were documented in Tanzania (SIVcpzTAN2 to SIVcpzTAN5) and around Kisangani in the north-east of DRC (SIVcpzDRC1). All the new SIVcpz*Pts* viruses formed a separate lineage together with the initially described SIVcpzANT strain. This indicated that the SIVcpz*Ptt* and not the SIVcpz*Pts* strains were at the origin of HIV-1 [321, 416]. Since the three groups of HIV-1 (M, N and O) fall within the HIV-1/SIVcpz*Ptt* radiation, the cross species transmissions

giving rise to HIV-1 most likely occurred in western equatorial Africa, within the range of *P.t.troglodytes* chimpanzees [122]. Furthermore, no human counterpart is found for SIVcpz*Pts* from *P.t.schweinfurtii*, ranging in East Africa. Four subspecies of chimpanzees are recognised on the basis of mitochondrial DNA sequence differences [134, 139, 247]. No chimpanzee from the western subspecies *Pan troglodytes verus* or the Nigerian/Cameroonian *Pan troglodytes vellerosus* was found to be infected with SIVcpz [183, 257, 384].

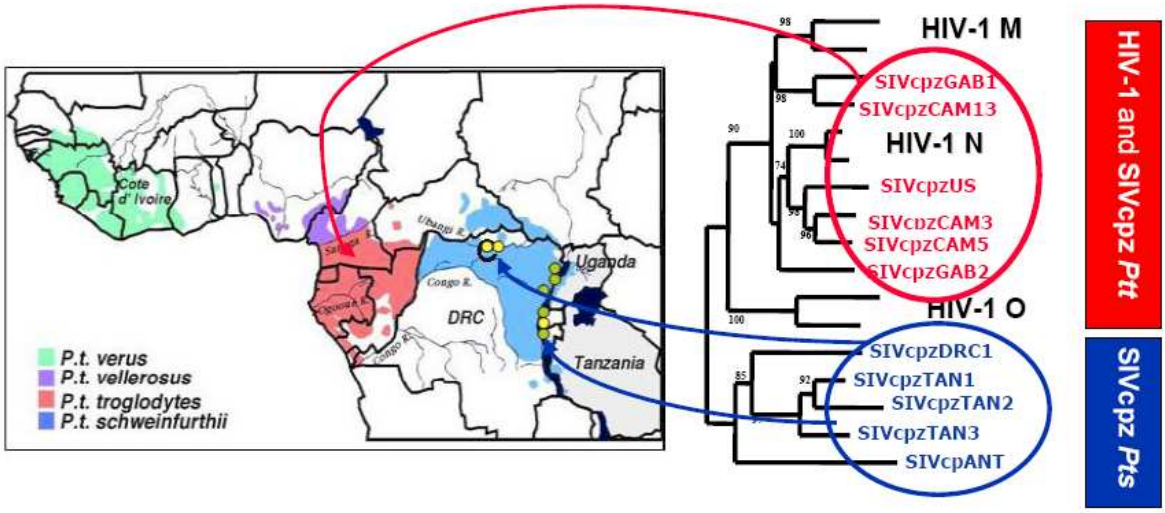



Figure 1.9: Evolutionary relationship of the SIVcpz/HIV-1 lineage. This indicates that HIV-1 is more closely related to SIVcpz*Ptt* from West-central African chimpanzees (red area) then the second SIVcpz lineage, SIVcpz*Pts* from the eastern chimpanzees (blue area). The tree was derived by the neighbour joining analysis of gp41/nef (~800bp) (Courtesy of Beatrice Hahn).

A recent study conducted in southern Cameroon documented a prevalence of SIV infection in chimpanzees ranging from 4% to 35%, based on 400 faecal samples collected. The 16 new SIVcpz*Ptt* strains identified fell within the radiation of SIVcpz*Ptt* strains from captive *P.t.troglodytes*. This radiation includes also HIV-1 groups M and N, but not group O or SIVcpz*Pts*. These new SIVcpz*Ptt* viruses are characterised by a high genetic diversity, and the SIVcpz*Ptt* identified were much more closely related to HIV-1 groups M and N than any previously identified SIVcpz strains.

Extension of the survey in Cameroon to a second great ape species, the western lowland gorillas (*Gorilla gorilla gorilla*), showed that they are also endemically infected

with a simian immunodeficiency virus, designated SIVgor [386]. The phylogenetic relationships between HIV-1, SIVcpz and SIVgor indicate that the gorilla viruses form a monophyletic lineage within the SIVcpz*Ptt* radiation, which is much more closely related to HIV-1 group O than to any other SIV (Figure 1.10). Although not yet detected in chimpanzees, the SIVgor virus seems to have a *P. t. troglodytes* origin, and it remains to be determined whether chimpanzees transmitted HIV-1 group O-like viruses to gorillas and humans independently, or first to gorillas, which then transmitted the virus to humans.

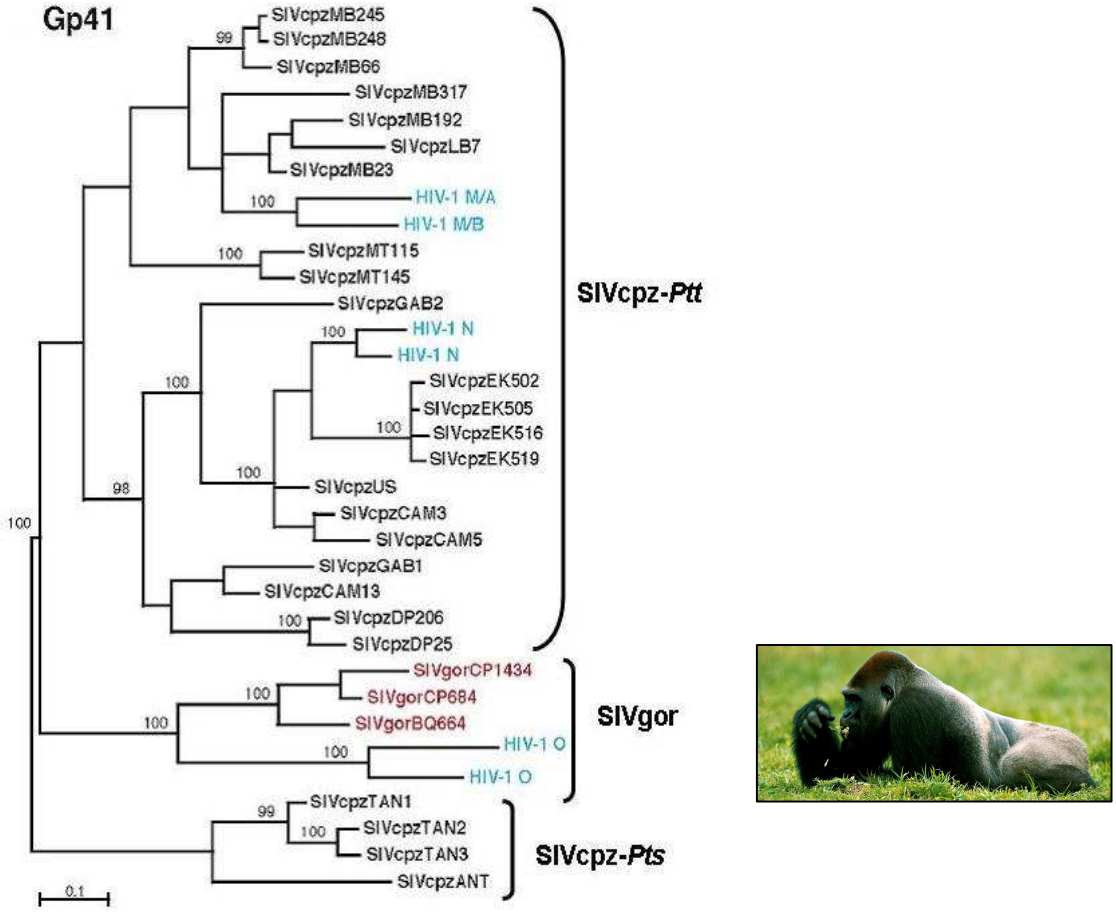



Figure 1.10: Evolutionary relationships of SIVgor (red), the three groups of HIV-1 (blue), SIVcpz-*Ptt* (*Pan troglodytes troglodytes*) and SIVcpz-*Pts* (*Pan troglodytes schweinfurthii*) of partial Env sequences, analysed by the Bayesian method. Posterior probability values above 95% are shown. Adapted from [386].

Picture 1.2: western lowland gorilla

### 1.2.3 Cross-species transmissions: where, when and how

The cross-species transmissions giving rise to HIV-1 (M, N, and O) most probably occurred in West-equatorial Africa, home of the *P.t.troglodytes* chimpanzee and the western gorilla infected with SIVcpz*Ptt* and SIVgor respectively. The recent studies on wild-living chimpanzees from Cameroon indicated that HIV-1 group M and N arose from geographically distinct chimpanzee's populations. Phylogenetic analysis of SIVcpz strains from samples collected in southeast Cameroon formed a cluster with HIV-1 group M, whereas SIVcpz strains from a well defined region in South-central Cameroon clustered with HIV-1 group N [183]. The recent identification of HIV-1 group O-like viruses in two separated wild-living gorilla populations in southern Cameroon strengthen the evidence of the seeding of HIV-1 in West-central Africa. In contrast to HIV-1 group N infections which remained restricted to Cameroon, and group O infections restricted to Cameroon, Gabon, and Equatorial guinea, group M strains have spread across Africa and all the other continents [147, 234]. The highest genetic diversity, in number of co-circulating subtypes and intrasubtype diversity, has been observed in the western part of DRC (Democratic Republic of Congo), suggesting that this region is the epicentre of HIV-1 group M [177, 394].

As already mentioned in sub-chapter 1.2.1, a close phylogenetic relatedness is observed between SIVsmm from sooty mangabeys (*Cercocebus atys*) and HIV-2 in West Africa. West Africa, from Senegal to Ivory Coast, is home to sooty mangabeys, coinciding with the endemic centre of HIV-2.

When did these cross-species transmissions occur? Molecular evolutionary analyses provided suitable information to answer this question. Dating the most recent common ancestor (MRCA) within each HIV group provides an upper limit on the time-scale for the estimation of the cross species transmissions. Molecular clock analyses estimated the date of the most recent common ancestor of HIV-1 group M at 1931 with a confidence interval (CI) of 1915 to 1941 [188, 319]. A similar time frame is estimated for the HIV-1 group O radiation; 1920 with a CI of 1890 to 1940 [211]. These time frames are congruent with the two historically documented points of the earliest known HIV-1 group M virus (subtype D), ZR59, isolated from an individual in Leopoldville (now

Kinshasa) in 1959 [425] and group O virus, identified in a Norwegian sailor in 1961, infected in Cameroon (Douala) [109]. Since the first identification of HIV-1 group N in 1998 [345], less than 10 group N infections have been described and all were from Cameroonian patients. The intra-group genetic diversity is significantly lower for group N than for group M or O, which suggests a more recent introduction of the HIV-1 N lineage into the human population.

Molecular clock analysis traced the origin of the HIV-2 groups A and B epidemic around 1940 (CI ± 16 years) and 1945 (CI ± 14 years), respectively [212].

Although the conditions and circumstances of SIVcpz and SIVsmm transmissions remain unknown, cutaneous or mucous membrane exposure to blood or other secretions, through hunting, butchering, biting or other injuries caused by infected NHPs, represents the most plausible source of human infection [122, 143]. The particular social, economic, and behavioural changes that occurred in the early and mid-20th century provided the circumstances whereby these viruses could expand and reach epidemic proportions.

### 1.2.4 The attenuated Oral Polio Vaccination (OPV) hypothesis

The attenuated Oral Polio Vaccination (OPV) hypothesis claims that HIV-1 was introduced into the human population iatrogenically through SIVcpz contamination of oral polio vaccines used in the vaccination programs that took place in Central Africa between 1957 and 1960 [159, 160]. Evidence presented today is all against the OPV hypothesis and should finally lay this theory to rest.

a. Material recovered or retained from early clinical trials in the Congo was examined by PCR in independent laboratories with negative results for SIV/HIV RNA and for chimpanzee cellular DNA. Positive results were obtained for macaque monkey cellular DNA [26, 30, 301].
b. In the 1950's, the same lots of OPV were fed to humans in Europe, without subsequent HIV infection [298].
c. Recovery of SIVcpz strains from chimpanzees in Africa showed that the chimpanzees available to the research team in the late 1950, had they been

infected with SIV, would have been infected by strains distant from HIV-1 because they belong to the subspecies *Pan troglodytes schweinfurthii*. SIVcpz strains that are close relatives to HIV-1, by contrast, belong to the subspecies *Pan troglodytes troglodytes* which is found in West-equatorial Africa, near the metropolis of Kinshasa where the first known HIV infection was identified [143].

d. For the OPV hypothesis to be considered, not only the kidneys used would have needed to be from the *P.t.troglodytes* subspecies, but also at least ten genetically distinct viruses would have had to enter the human population through the vaccine. As SIVcpz infections are rare in captive animals (data derived from hundreds of animals suggest a prevalence of roughly 1% [73, 122, 288, 290]), and a small number of primate kidneys were needed for OPV cultures [160], this seems implausible.

e. Furthermore, the young age of the captured animals used for research in the facility close to Stanleyville (present-day Kisangani) [160] is also inconsistent with the hypothesis that the HIV-1 subtypes resulted from the introduction of diverse quasispecies infecting the kidney of a single animal, because the distance between the subtype progenitors exceeds typical intrahost variation, particularly in infants and juveniles.

f. Finally and most importantly, the M group of HIV-1 has been estimated to have originated 10 to 50 years before the OPV vaccine trials were conducted [188].

## 1.3 The Simian Immunodeficiency Viruses (SIVs)

In the previous section I described the origin of HIV-AIDS in three different NHP species. To date, SIV has been isolated not only in certain anthropomorphic primates (i.e., chimpanzees, gorillas) or in the Papionini tribe of the Cercopithecinae (i.e., sooty mangabeys), but also in Cercopithecines and Colobines, for a comprehensive total of 40 species of African primates [382, 387]. SIVs are followed by a three letter code

indicating the primate species in which the virus was isolated: for example SIVagm has been isolated in African Green Monkeys (for a comprehensive list of SIV nomenclature, please refer to Table 1.1).

### 1.3.1 SIV diversity relative to viral genomic structure

Using gene and open reading frame (ORF) structures as a method for determining relatedness, three groupings of SIVs can be identified. All primate lentiviruses harbour five regulatory genes (*vif*, *rev*, *tat*, *vpr*, and *nef*) that generally fall in the same regions of the SIV/HIV genome. *tat* and *rev* each consist of two exons. The presence of two other regulatory genes (*vpx* and *vpu*) is variable and thus defines three patterns of genomic organisation, as follows (Fig. 1.11):

(i) SIVsyk, SIVdeb, SIVasc, SIVtal, SIVagm, SIVmnd-1, SIVlhoest, SIVsun, and SIVcol contain only five accessory genes (*tat*, *rev*, *nef*, *vif*, and *vpr*) [20, 21, 27, 78, 83, 151, 153, 154, 215].

(ii) HIV-1, SIVcpz, SIVgor, SIVgsn, SIVmus, SIVmon, and SIVden genomes include a supplementary gene, *vpu* [76, 87, 165]. HIV-1 and SIVcpz differ from the other members of this group by the fact that *env* and *nef* genes are not overlapping.

(iii) HIV-2, SIVsmm, SIVmac, SIVrcm, SIVmnd-2, and SIVdrl form the third genomic group, which is characterised by the presence of the *vpx* gene [23, 56, 155, 162, 350]. Thus far, *vpx* appears to be specific for SIVs infecting the Papionini group of monkeys and was acquired following a nonhomologous recombination, which resulted in a duplication of the *vpr* gene [335].

(iv) SIVblu [27], SIVbkm [366], SIVpre, SIVery, and SIVagi cannot be characterised by gene organisation at this time, since the complete genome sequences are not yet available.

(v) SIVwrc and SIVolc have been only partially characterised in the past [77]. Their full-length genome characteristics are described in the frame of this thesis.

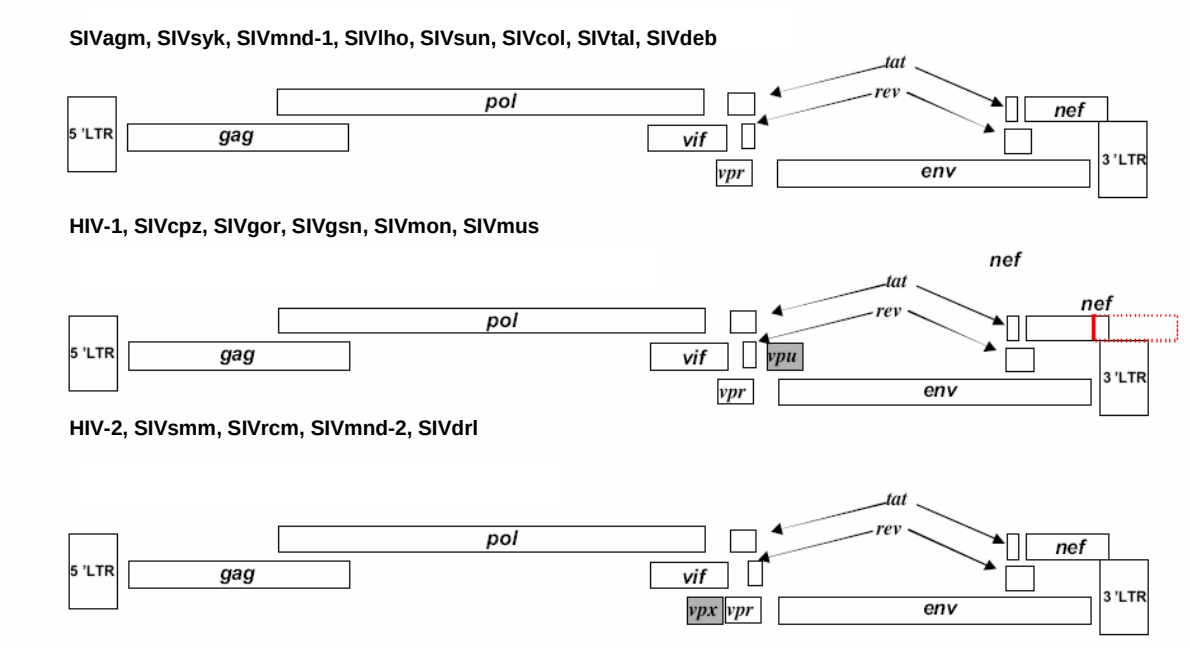



Figure 1.11: Genomic organisation of primate lentiviruses

### 1.3.2 SIV natural hosts and SIV genetic diversity

Initially, the different SIVs identified, which were forming separate phylogenetic clusters, were grouped in six lineages. Classically, the following six such lineages have been described:
(i) SIVcpz/HIV-1, (ii) SIVsmm/HIV-2, (iii) SIVagm, (iv) SIVsyk, (v) SIVlho, (vi) SIVcol [22, 78, 337].

When phylogenetic analyses were performed on different genomic regions, the virus classification became complicated because the sequences' diversity and observed recombination resulted in different patterns of clustering.

With the characterisation of new SIV strains, it became clear that the identification of "pure lineages" or "recombinants" was primarily a matter of chronology and it was suggested that each of the "classical" lineages may be in fact recombinant [318]. Some of the classical lineages were indeed shown to be formed by recombinant strains, with the most notable being the SIVcpz/HIV-1 lineage.

The remaining “non-recombinant” strains cluster into six lineages. These six phylogenetic lineages are approximately equidistant, with genetic distances of up to 40% in Pol proteins (Figure 1.12 ).

The SIVlho lineage is unique in being formed by SIVs circulating in distantly related species. The relationship between these SIV lineages and newly characterised SIVs is complex, such that the characterization of recombinants is limited to the most obvious mosaic genomes.

A more recent classification based upon phylogenetic relationships lists the known SIVs in the following clusters [382]:

1. Arboreal guenons (Cercopithecus): SIVsyk, SIVblu, SIVgsn, SIVdeb, SIVmon, SIVden, SIVmus, SIVasc, SIVtal, SIVery
2. Sooty mangabey: SIVsmm
3. African green monkey: SIVagm (SIVagm.ver, SIVagm.tan, SIVrcm.gri, SIVagm.sab)
4. L’Hoest supergroup, mandrill: SIVlho, SIVsun, SIVmnd-1
5. Red-capped mangabeys: SIVrcm, SIVagi
6. Mantled guereza: SIVcol

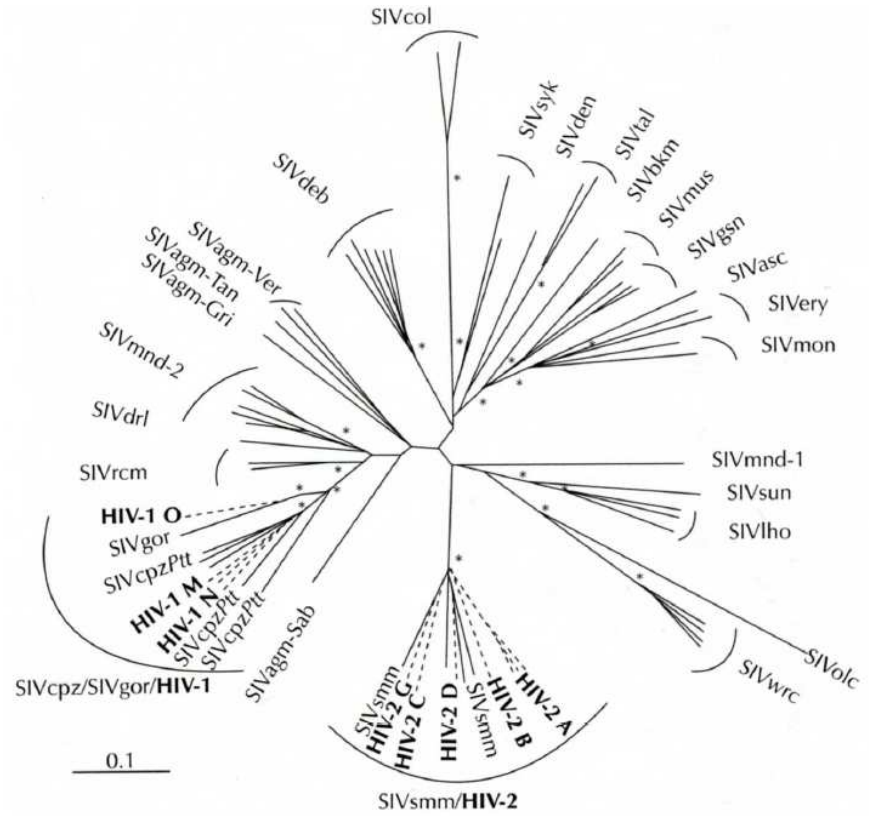



Figure 1.12: Evolutionary relationship among the different simian immunodeficiency viruses (SIV) and HIV lineages based on neighbour joining phylogenetic analysis of partial *pol* sequences. The consensus length of the final alignment used for tree construction was 527 bp. Branch lengths are drawn to scale (the scale bar indicates 0.1 substitutions per site). *Asterisks* indicate bootstrap replicates supporting the cluster to the right with values >85%.This phylogenetic tree includes 26 of the 32 infected NHP species for whom partial sequences are available. The different HIV-1 and HIV-2 lineages interspersed with the SIVcp/SIVgor and SIVsmm lineages, respectively, are indicated in bold (Courtesy of F. Van Heuverswyn).

### 1.3.2.1 Virus-host co-evolution, cross-species transmissions and recombination between virus species

Phylogenetic analyses of the available SIV strains have shown a high level of genetic diversity and a starburst phylogenetic pattern suggesting evolution from a single ancestor (Figure1.13). Asian species of Old World monkeys (colobines and macaques) have not found to be naturally infected with SIVs, which has also suggested that the last common ancestor of the catarrhines (Old World monkeys and apes) was not infected by

SIV 25 million years ago [22]. This suggests that the emergence of SIV followed infection after radiation of these species, possibly from a non-primate source [333].

The approximate equidistance among the major SIV lineages does not always match the relationships between their hosts. As depicted in Figure 1.13, the SIV phylogenetic clusters are only partially superimposable on primate phylogenetic trees.

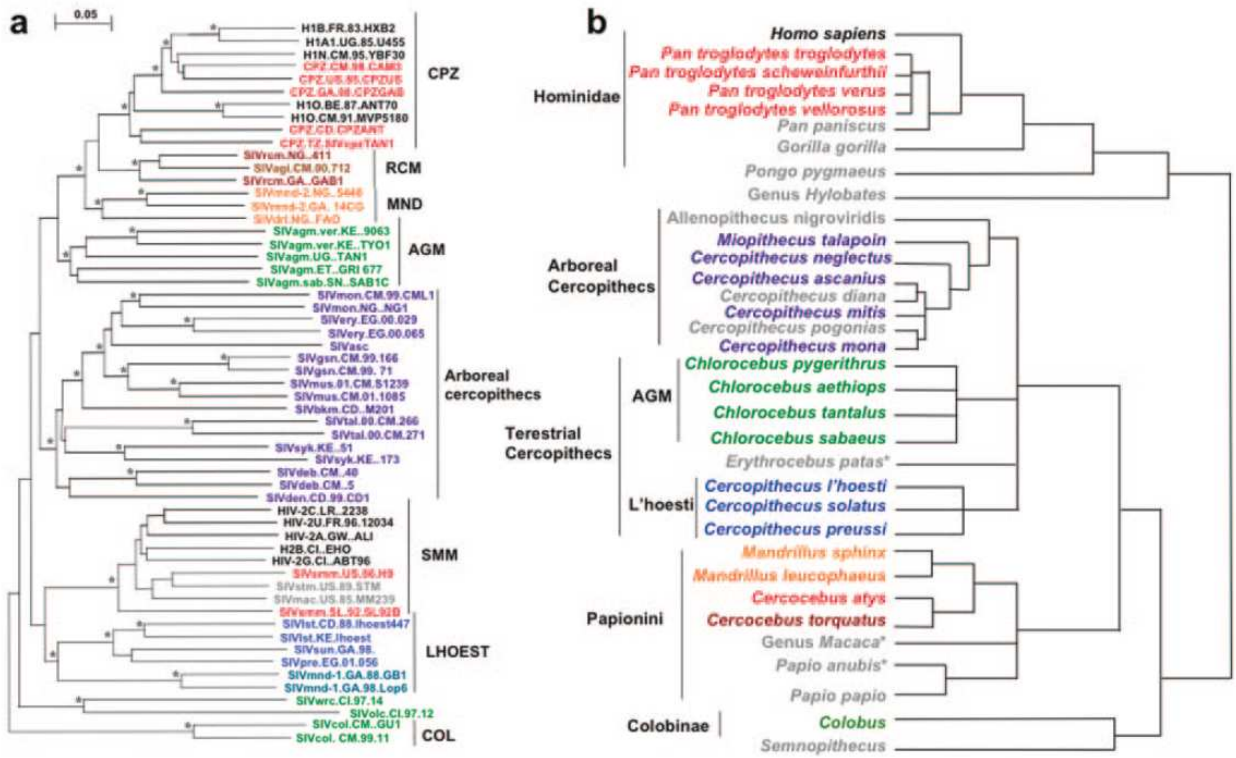



Figure 1.13: Comparison between SIV phylogeny (a) and primate phylogeny (b)
(a) Neighbour-joining tree constructed from available SIV sequences; (b) primate phylogeny is shown by a schematic relationships cited in the text. While general alignment of hosts and viruses can be observed, cross-species transmissions and viral recombination events make this correlation less than absolute. Asterisks indicate significant bootstrap values. Note that at that time the SIVgor in *Gorilla gorilla gorilla* was not discovered. Source: [382].

There are cases where virus phylogeny seems to correspond to host phylogeny, such as SIVs infecting the four African green monkey species for example. However, the hypothesis that SIVagm co-evolved with their hosts has been recently challenged [402].

Further characterisation of SIVs showed that several variants have discordant phylogenies when different genes are studied. For example SIVagm-Sab isolated from sabaeus monkeys has a mosaic genome structure, with a *gag-pol* insert with a SIVrcm structure [168]. SIVrcm from red capped-mangabeys (*Cercocebus torquatus*) is closely related to SIVagm-Sab in *gag*, to SIVcpz in *pol-vif*, and to SIVsmm in *env-nef* [23]. SIVmnd-2 and SIVdrl isolated from mandrills (*Mandrillus sphinx*) and drills (*Mandrillus leucophaeus*) respectively are related to SIVrcm in *gag*, *pol*, *vif*, *vpx* and tat and to SIVmnd-1 in *env-nef* [162, 350].

Another example of SIV recombination is that of SIVcpz. The 5' region of SIVcpz (*gag, pol, vif* and *vpr*) is most similar to SIVrcm from red capped mangabeys [23], except for the accessory gene *vpx*, which is characteristic for the SIVrcm lineage, but absent in the HIV-1 and SIVcpz strains, whereas in the 3' region of its genome (*vpu, env* and *nef*) SIVcpz is found to be closely related to a SIVgsn lineage including greater spot-nosed monkeys, mustached monkeys and mona monkeys [16, 76, 79]. Because chimpanzees are known to hunt other primates for food, such as colobus monkeys (*Colobinae*), especially red colobus (*Piliocolobus badius*), mangabeys (*Lophocebus albigena*), blue monkeys (*Cercopithecus mitis*), but also greater spot-nosed monkeys (*Cercopithecus nictitans*) and red-capped mangabeys (*Cercocebus torquatus*) [31, 351, 356], the most obvious explanation is that chimpanzees acquired SIV infection from other monkeys rather than the opposite. The recombination of these monkey viruses must have occurred within chimpanzees and given rise to the common ancestor of today's SIVcpz lineages, which in turn were subsequently transmitted to humans [14].

These examples indicate that cross-species transmissions and recombination have occurred among different species of NHPs. Subsequently, SIVs can evolve in different ways in their new hosts and could further recombine and spread to other NHPs. Data on NHP behavioural ecology, past and present geographic distribution and

evolution can help to determine which viruses may represent pure lineages and which may be recombinants.

Evidence of cross-species transmission of SIVagm has been observed in the wild, where this virus has been isolated from a yellow baboon (*Papio cynocephalus*) [169], a chacma baboon (*Papio ursinus*) [390], and a patas monkey (*Erythrocebus patas*) [29]. However, systematic prevalence studies have not been carried out yet to determine if SIVagm is established as a virus endemic to these species.

It has been observed that arboreal guenons are infected with viruses sharing biological properties and structural features and forming a single cluster. The *Cercopithecus* genus regroups the highest number of species identified so far to be infected with SIV. SIV from SIVsyk, SIVdeb, SIVgsn, SIVmus and SIVmon not only cluster together in phylogenetic trees, but they also share functional motifs in their genome that distinguish them from other primate lentiviruses [27]. Partial sequence analysis of SIVs from several other guenons like SIVery (*C. erythrotis*), SIVasc (*C. ascanius*) , SIVblu (*C. mitis*), SIVpre (*C. preussi*), and SIVden (*C. denti*) fall within the *Cercopithecus* cluster leading to the proposition of some authors to regroup all these lentiviruses in a *Cercopithecus* monkey virus lineage [27, 87, 393].

The branching of two *Cercopithecus* viruses, SIVlho and SIVsun, outside of the *Cercopithecus* cluster can probably be explained by a misclassification of the two species in the *Cercopithecus* genus. Recent studies indicate that SIVlho and SIVsun should no longer be classified as *Cercopithecus* monkey viruses, but should be considered as a separate group more closely related to the terrestrial guenons of the *Chlorocebus* and *Erythrocebus* genera [376].

SIVtal isolated in talapoins share some functional motifs of the *Cercopithecus* SIV and cluster with them. However, talapoins' taxonomy is controversial and classification in the *Cercopithecus* or in the *Miopithecus* genus is unsolved.

SIVbkm, isolated from a black mangabey, falls within the SIV lineage of the *Cercopithecus* monkey viruses, but for this strain only a small part of the *pol* gene is available, so no additional data is available to support its phylogenetic position [366].

Papionini monkeys (*Cercocebus* spp.) are infected with related viruses, although recombinant viruses can be observed in these monkeys [162].

The case of the mandrills shows that one primate species can be infected with two different SIVs. The distribution of the two types of SIVmnd is geographically separated by the Ogooué River in Gabon. SIVmnd-1 viruses were exclusively identified in mandrills from central and southern Gabon, whereas SIVmnd-2 was isolated in monkeys from northern and western Gabon and in Cameroon [350, 364]. The southern type-1 virus may have originated from a cross species transmission from *C. solatus* or a C.*lhoesti*/*solatus* ancestor, while the northern type-2 virus appears to be the result of the recombination between an ancestral mandrill–drill virus with SIVrcm from *C. t. torquatus* [127, 350].

Another example of a single monkey species which harbours two distinct SIV lineages is the moustached monkey (*Cercopithecus cephus*), which is infected with SIVmus-1 and SIVmus-2. These two virus variants are not geographically separated, but co-circulate instead in monkeys sharing the same habitat in the same geographic area in southern Cameroon [1]. These two viruses form two distinct groups within the clade comprised of lentiviruses isolated from *C. nictitans* (SIVgsn), *C. mona* (SIVmon) and *C. cephus* (SIVmus). Phylogenetic analyses showed that the diversification of SIVmus, SIVgsn and SIVmon involved inter-lineage recombination, and suggested that one of the SIVmus lineages likely resulted from cross-species transmission and recombination involving SIVmus and an as yet uncharacterised SIV.

Cross-species transmission events can be expected to occur among co-habiting primates for whom contacts (through predation, habitat or food competition or sexual contacts) between different species have been documented. For instance, SIVlho, SIVsun and SIVmnd-1 have been isolated from three different species, the l'Hoest monkey (*Cercopitheccus lhoesti*), the sun-tailed monkey (*Cercopitheccus solatus*), and the mandrill (*Mandrillus sphinx*). Sun-tailed monkeys and l'Hoest monkeys, together with Preuss's monkeys (*Cercopitheccus preussi*) are the 3 species of the super-species l'hoesti from the *Cercopithecini* tribe, whereas mandrills represent a genus from the *Papionini* tribe [139]. The range of mandrills and sun-tailed monkeys overlaps in West-equatorial Africa, whereas l'Hoest monkeys are common in East Africa.

Nevertheless, SIVlho and SIVsun are more closely related to each other than to SIVmnd. This reflects on one side an example of host dependent evolution [21]; on the other side, the presence of quite closely related viruses (SIVsun/SIVlho and SIVmnd-1) in quite distantly related hosts (guenons and mandrills) suggests that SIVmnd-1 resulted from a cross-species transmission of SIVsun from sun-tailed monkeys in Gabon [367].

The phylogenetic analysis conducted so far on colobines showed that partial viral sequences from western red (SIVwrc) and olive (SIVolc) colobus each formed species specific monophyletic clusters. They were more closely related to each other than to the other SIVs and were not at all related to the SIVcol strain obtained from a mantled guereza (*C.guereza*) from Cameroon [77] . By looking only at these partial sequences, it was hypothesized that SIVcol, SIVwrc, and SIVolc did not evolve in a host-dependent fashion at the level of the Colobinae subfamily. In fact, the representatives of these 3 genera were not clustering together in the genomic region studied. Full length characterisation of SIVwrc and SIVolc are needed to confirm this and will be discussed further on, in the frame of this thesis.

Finally, in phylogenetic trees, HIV-1 and HIV-2 are dispersed among related SIVs and show no species-specific pattern. Thus, from a phylogenetic point of view, the differentiation between HIVs and SIVs is irrelevant, which is the basis for the argument supporting the simian origin of HIV [332, 334, 336].

In conclusion, the genetic diversity of NHP lentiviruses reflects a complex evolutionary history, which has been driven by host-virus co-speciation, cross-species transmission and recombination over an extended period of time. By characterising additional NHP lentiviruses, new viral/host relationships could be revealed and the extent of past or recent cross-species transmission, superinfection and recombination could be further elucidated.

## 1.4 SIV epidemiology

### 1.4.1 Prevalence

Among the 40 NHP species which have been recognised, to date, to be infected with SIV, 9 have been confirmed only by serological evidence, whereas for 24 species, at least one complete genome has been sequenced. Table 1.1 illustrates an update of the African NHP species infected with SIV, their geographic distribution and the results obtained (full length genomes, partial sequences or serological evidence).

Prevalence results vary according to the methods applied (serological versus molecular), the sampling size, and according to whether sampling was performed on wild-living populations or on captive animals living in zoos or research centres. As a consequence, prevalence data are not comparable.

For example, past studies of SIV in chimpanzees reported a very low prevalence, probably because primarily captive juveniles were tested. More recent sero-epidemiological studies conducted in West-equatorial and East Africa reported a prevalence of up to 35% of SIVcpz in *Pan troglodytes troglodytes* and in *P.t.schweinfurthii* subspecies respectively [183, 321]. Similarly, in Cameroon, the prevalence of SIVgsn, the virus that naturally infects greater spot-nosed monkeys (*Cercopithecus nictitans*), was established at 16% (27/165) [79]. However, when a significant number of samples were tested using specific SIVgsn peptides, prevalence rates of 4% were reported [2].

Due to their number, genetic diversity, and large distribution in sub-Saharan Africa, guenons (tribe Cercopithecini) are the largest reservoir species for SIV, as shown in Table 1.1. I invite the reader to refer to a recent review on SIV genetic diversity for further details on SIV prevalence [382]. The prevalence of SIVwrc in wild-living western red colobus and the molecular characteristics of SIVwrc and of SIVolc from olive colobus (appearing in bold in Table 1.1) will be discussed further on in the frame of this thesis.

Table 1.1: African NHPs infected with SIV: an update.

| ***Genus*** | ***Species*** | **Common name** | **Virus strain** | **Geographic distribution** | **Available sequences** |
|---|---|---|---|---|---|
| *Pan* | *troglodytes* | Chimpanzee | SIVcpz | West to East: Senegal to Tanzania | CG + PS |
| *Gorilla* | *gorilla* | Lowland gorilla | SIVgor | West Central: from the river Cross to rivers Shanga/Congo | CG |
| *Colobus* | *guereza* | Mantled guereza | SIVcol | Central: Nigeria to Ethiopia/Tanzania | CG + PS |
| ***Piliocolobus*** | ***badius*** | **Western red colobus** | **SIVwrc** | **West: Senegal to Ghana** | **CG + PS** |
| ***Procolobus*** | ***verus*** | **Olive colobus** | **SIVolc** | **West: Sierra-Leone to Ghana** | **CG** |
| *Lophocebus* | *albigena* | Gray-cheeked mangabey mmangabeymmamangabey | ? | Central: Nigeria to Uganda/Burundi | Serological evidence |
| | *aterrimus* | Black crested mangabey | SIVbkm | Central: Democratic Republic of Congo (DRC) | Serological evidence |
| *Papio* | *anubis* | Olive baboon | ? | West to East: Mali to Ethiopia | Serological evidence |
| | *cynocephalus* | Yellow baboon | SIVagm-ver | Central: Angola to Tanzania | PS |
| | *ursinus* | Chacma baboon | SIVagm-ver | South: Southern Angola to Zambia | PS |
| *Cercocebus* | *atys* | Sooty mangabey | SIVsmm | West: Senegal to Ghana | CG + PS |
| | *torquatus* | Red-capped mangabey | SIVrcm | West Central: Nigeria, Cameroon, Gabon | CG + PS |
| | *agilis* | Agile mangabey | SIVagi | Central - Northeast Gabon to northeast Congo | PS |
| *Mandrillus* | *sphinx* | Mandrill | SIVmnd-1, SIVmnd-2 | West Central: Cameroon (south of Sanaga river) to Gabon, Congo | CG + PS |
| | *leucophaeus* | Drill | SIVdrl | West Central: Southeast Nigeria to Cameroon (north of Sanaga) | CG + PS |
| *Allenopithecus* | *nigroviridis* | Allen's swamp monkey | ? | Central: Congo | Serological evidence |
| *Miopithecus* | *talapoin* | Angolan talapoin | SIVtal | West Central: East coast of Angola into DRC | CG + PS |
| | *ogouensis* | Gabon talapoin | SIVtal | West Central: Cameroon (south of Sanaga river) to Gabon | CG + PS |
| *Erythrocebus* | *patas* | Patas monkey | SIVagm-sab | West to East: Senegal to Ethiopia, Tanzania | PS |
| *Chlorocebus* | *sabaeus* | Green monkey | SIVagm-sab | West: Senegal to Volta river | CG + PS |
| | *aethiops* | Grivet monkey | SIVagm-gri | East: Soudan, Erithrea, Ethiopia | CG + PS |
| | *tantalus* | Tantalus monkey | SIVagm-tan | Central: Ghana to Uganda | CG + PS |
| | *pygerythrus* | Vervet monkey | SIVagm-ver | South: South Africa to Somalia and Angola | CG + PS |
| *Cercopithecus* | *diana* | Diana monkey | ? | West: Sierra-Leone to Côte d'Ivoire | Serological evidence |
| | *nictitans* | Greater spot-nosed monkey | SIVgsn | Central: forest blocks from West Africa to DRC | CG + PS |
| | *mitis* | Blue monkey | SIVblu | East Central: East Congo to Rift-valley | PS |
| | *albogularis* | Sykes's monkey | SIVsyk | East: Somalia to Eastern Cape | CG + PS |
| | *mona* | Mona monkey | SIVmon | West: Niger delta to Cameroon (North of Sanaga) | CG |
| | *lowei* | Lowe's monkey | ? | West: Liberia to Côte d'Ivoire | Serological evidence |
| | *campbelli* | Campbell's monkey | ? | West: Gambia to Liberia | Serological evidence |
| | *pogonias* | Crested mona | ? | West Central: Cross-river in Nigeria to Congo (east) | Serological evidence |
| | *denti* | Dent's mona | SIVden | Central: south of Congo river, DRC | CG |
| | *cephus* | Mustached monkey | SIVmus | West Central: Cameroon (south of Sanaga) to east of the Congo river | CG + PS |
| | *erythrotis* | Red-eared monkey | SIVery | West Central: Cross river in Nigeria to Sanaga in Cameroon, Bioko | PS |
| | *ascanius* | Red-tailed monkey | SIVasc | Central: South-East Congo to West Tanzania | CG + PS |
| | *lhoesti* | L'Hoest monkey | SIVlho | Central: eastern DRC to western Uganda | CG + PS |
| | *solatus* | Sun-tailed monkey | SIVsun | West Central: tropical forest of Gabon | CG + PS |
| | *preussi* | Preuss monkey | SIVpre | West Central: Cross river in Nigeria to Sanaga in Cameroon, Bioko | PS |
| | *hamlyni* | Owl-faced monkey | ? | Central: eastern DRC to Ruanda | Serological evidence |
| | *neglectus* | De Brazza monkey | SIVdeb | Central: Angola, Cameroon, Gabon to Uganda, Western Kenya | CG + PS |

CG: Complete genome; PS: partial sequences

### 1.4.2 Routes of transmission

Sexual intercourse seems to be the most prevalent mode of transmission of simian immunodeficiency virus. Sero-epidemiologic surveys of African green monkeys (AGMs), sooty mangabeys (SMs), and mandrills (MNDs) revealed higher prevalence levels for adult monkeys than for juveniles, indicating a horizontal route of transmission [91, 295]. However, in a semi-free colony of MNDs at the International Medical Research Centre of Franceville, no sexual transmission was found after 16 years of follow-up [71, 101, 128, 350]. SIVmnd-1 was transmitted to four offspring (males and females) of the SIVmnd-1-infected female founder. SIVmnd-2 was transmitted from the infected male founder to four other males following aggressive contacts for dominance [256]. Several cases of horizontal transmission occurring by biting have been described for captive monkeys, including AGMs [295], SMs [312], and chimpanzees [73]. SIVsmm has been reported to be transmitted to macaques by biting [222]. SIV vertical transmission seems to be less frequent than horizontal transmission, and the point of transmission (in utero, perinatally, or via breast milk) has not been identified yet. In a prospective study, experimental mother-to offspring transmission by breast-feeding was not observed in MNDs (I. Pandrea, unpublished results cited in [382]), while another study did not demonstrate vertical transmission in AGMs [278]. Conversely, phylogenetic results suggested vertical transmission as a potential mechanism of SIVsmm transmission [7, 322].

## 1.5 SIV pathogenecity

### 1.5.1 Pathogenicity of African Primate SIV infection

Despite active viral replication and high prevalence levels, SIV infections are generally non-pathogenic in their natural hosts (for a review see Vande Woude and Apetrei, 2006). Progression to AIDS is a rare event, but can occur when monkeys are significantly older than the mean life span and when animals have been infected over

long periods of time. For example, 2 mandrills infected with SIVmnd-1 and SIVmnd-2 developed AIDS, after 17 years of SIV infection [283]. A sooty mangabey naturally infected with SIVsmm progressed to AIDS after an incubation period of 18 years. An AGM coinfected with SIVagm and simian T-cell leukaemia virus (STLV) was also reported to progress to AIDS [377]. In every case, AIDS was supported by biological signs as increased viral replication, weight loss, opportunistic infections and lymphomas.

AIDS was reported to develop in African NHPs after infection with heterologous viruses. The first compelling evidence occurred during a leprosy experiment where three black mangabeys were inoculated with lepromatous tissue that had been serially passaged in SIV-positive SMs. Clearance of infection occurred in two out of three animals, and the third developed AIDS after 5 years [5]. Baboons, which are not known to be infected naturally with SIV, developed AIDS following infection with HIV-2 [17]. A few chimpanzees also developed AIDS after having been infected several times with HIV-1 [261, 268]. However, in a recent study addressing the question of SIVcpz transmission, a cohort of 9 captive bred chimpanzees was infected with SIVcpz. None of them developed AIDS despite coinfection with HIV-1 in six of them [146].

There is currently a consensus that understanding the mechanisms that contribute to the lack of SIV disease progression in African NHPs will represent a major advancement for designing new approaches to control HIV. Pathogenesis studies are limited in African NHPs because most of these SIVs are only known from genomic sequences; moreover, numerous African NHP species are endangered and therefore difficult to use for these studies. Moreover, monkey importation from Africa is limited. Three models have been developed thus far for the study of SIV pathogenesis in African hosts: SIVagm in African green monkeys (AGMs), favoured by the wide availability of this unendangered species [92, 132, 190], SIVsmm infection in sooty mangabeys (SM), carried out in the large SM colony existing at the Yerkes National Primate Research Center in the USA [180, 181, 342, 344], and SIVmnd-1/SIVmnd-2 in mandrills (MND), favoured by the existence of a state-of-art research facility in the endemic area of mandrills, Franceville, Gabon [162, 350, 364]. These models have produced a better understanding of SIV infection in natural NHP hosts, particularly in relation to viral load and immune responses.

### 1.5.2 Viral load and immune responses in SIV infection

The early dynamics of SIV replication have been investigated by performing experimental SIV infections in all available models [92, 132, 190, 276, 284]. These studies revealed a massive viral replication during the primary infection, resulting in a transient CD4 depletion. Moreover, these studies revealed that there is only transient immune activation during the acute infection which is rapidly controlled during the chronic infection by compensatory mechanisms aimed to install an anti-inflammatory milieu in SIV African hosts [190, 276, 282, 343, 344]. Therefore, the current view is that the control of immune activation rather than that of viral replication is the major mechanism through which natural hosts are protected against disease. This is in striking contrast to the dramatic increases in immune activation levels observed in HIV-infected human patients and SIV-infected macaques [95, 344].

### 1.5.3 SIV Receptor Use and Tropism

Non-human primates naturally infected with SIV use the same repertoire of receptors used by HIV-1, namely CD4 as a binding receptor and chemokine coreceptors like CCR5 and CXCR4. Most of the SIVs naturally infecting African NHPs use CCR5 as the main coreceptor [64, 424]. In 50% of HIV-1 infection, the progression to AIDS is characterised by a switch in viral tropism from R5 (“macrophage” tropic) to X4 (“lymphocyte” tropic) viruses [244]. However, for NHPs, no correlation has been established between coreceptor usage and pathogenesis in vivo. For example, SIVmac, which is more virulent than HIV-1, is a R5 virus strain [424]. Conversely, SIV-mnd-1, SIVagm.sab, and some strains of SIVsmm use CXCR4 receptors, but no pathological correlation has been described [279, 282, 327]. Natural hosts for SIV infection, such as SMs, AGMs, MNDs, and chimpanzees, express lower levels of CCR5 on memory CD4+ T cells in PBMC and mucosal tissues than do immunodeficiency-susceptible hosts, such as macaques, baboons, and humans [281]. Since CCR5 has been shown to be the main coreceptor used by SIVs in natural hosts [424], African species with endemic naturally occurring SIVs may be less susceptible to pathogenic disease because they

have fewer receptor targets for infection [281]. However, this does not explain the fact that SIV VLs in apathogenic infections are equivalent to pathogenic levels. Differences in receptor usage or target cell receptor may differ from species to species and may have evolved over time to favour a more balanced host-virus relationship. Clearly, a simple association between the number of susceptible target cells and disease cannot be sustained.

### 1.5.4 Restriction factors

Recent discoveries have revealed that human (and non-human primate) cells harbour at least two intrinsic (or non-immune) intracellular resistance mechanisms that can suppress HIV-1 infection. The first is mediated by a member of the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) family of cytidine deaminases [225, 339]. The deaminase is incorporated into the lentiviral virion during reverse transcription to direct the deamination of cytidine to uridine on the minus strand of viral DNA. This deamination results in catastrophic G-to-A mutations in the viral genome, determining inactivation [144, 225] and/or degradation of the viral genome. Two primate APOBEC family members (APOBEC3G and APOBEC3F) are believed to play a central role in antagonizing viral replication because they are expressed in natural targets of HIV-1 infection, including lymphocytes and macrophages. However, lentiviruses are able to successfully infect and replicate in host target cells containing APOBEC when host-adapted viral Vif interferes with this mechanism. This Vif activity is species specific, i.e., human APOBEC3G is inhibited by HIV-1 Vif, but not by SIVagm Vif, whereas AGM APOBEC3G is inhibited by SIVagm Vif, but not by HIV-1 Vif [34, 328]. The mechanism of this reaction is complex and is still being investigated intensively.

The second intracellular resistance mechanism is mediated by TRIM (tripartite interaction motif) proteins and was revealed through studies of species-specific post-entry blocks to HIV and SIV infections [360]. It is believed that TRIM5α interferes with the viral uncoating step that is required to liberate viral nucleic acids into the cytoplasm upon viral binding and fusion with the target cell [131]. Trim5α genes show that each

one has a unique viral specificity, but in no case so far do they have substantial activity against retroviruses that currently endemically infect that species.

Because host populations evolve much more slowly than viral populations do, the recognition of a new virus will therefore always lag behind unless coincidental viral restriction already existed in the newly infected host. This means that the Trim5α that each species currently possesses protects that species from past infections (and possibly, serendipitously, future ones), but not current ones [325]. This is consistent with the hypothesis that today's SIVs infections are non pathogenic in their natural hosts, because they are representative of the population which survived the disease occurring in the distant past. Heterologous infections would still represent a threat, as confirmed by the consequences of cross-species transmissions of SIVcpz and SIVsmm into different primate species.

### 1.5.5 Experimental animal models for AIDS

For many years chimpanzees have been the model of choice to study HIV-1 infection and therapy. The chimpanzee model has been adopted for studies of virus transmission [130] and for small-scale studies of passive immunization and vaccine strategies [112, 252]. However, concern was raised over the relevance of these studies, since virulent infections were absent or rare; hence the utility of this model for pathogenesis studies was limited. Approximately 150 chimpanzees have been experimentally infected with HIV-1 and only four were reported to progress to AIDS [262, 268]. Additionally, ethical and budgetary reasons also contributed to the decrease of experiments of HIV-1 infection in captive chimpanzees.

Another animal model for the study of AIDS is that of the macaque infected with SIVsmm. CD4+ T cell loss is reported in infected animals and progression to AIDS occurs at intervals ranging from several weeks to several years. The SIVmac model has been used extensively for studies on pathogenesis, virus-host cell interaction and efficacy of antiretroviral drugs. Although the infection is pathogenic with progression to AIDS, SIVmac does not represent an ideal model to study HIV-1 infection in humans. The clinical course of SIV infection in macaques is much more aggressive than that of

HIV-1 infection in humans and it is characterised by a larger proportion of rapid progressors [403]. Furthermore, other strains of SIV (i.e. SIVcpz, SIVrcm, SIVagm, SIV mnd-2 and SIVsyk) induce a shorter and lower viral replication after inoculation in Rhesus macaques [19, 99, 172, 349, 364], therefore it is possible that the high pathogenicity of SIVsmm in several macaques species is the result of external factors which have turned the SIVsmm in a highly virulent strain instead of SIVsmm being intrinsically virulent [382].

To overcome the limitations of HIV-1 to cause infection in most chimpanzees, macaques and other NHP, chimeric simian human immunodeficiency viruses (SHIV) were created [340]. SHIV chimeras were created by inserting HIV-1 *env*, *rev*, *tat* and *vpu* genes into SIVmac sequences and were shown to induce AIDS after serial passages in macaques [170, 171, 317]. The SHIV model which incorporates the HIV-1 *env* gene has been created to evaluate the efficacy of vaccines directed against the HIV-1 envelope and for studies on passive immunization [197], whereas single genes or gene fragments exchanges are used to study the function of a particular gene or to test antiretroviral drugs. For example, the replacement of the reverse transcriptase of SIVmac by that of HIV-1 in a RT-SHIV/macaque model has allowed the investigation of the antiviral properties of most RT drug inhibitors [15, 260].

## 1.6 Diagnostic and identification strategies for SIVs

### 1.6.1 Methods of SIV investigation

It is imperative to census all NHP species for SIV infection to better characterise the extent and the genetic diversity of this virus. In Africa, at least 69 NHP species have been identified and only 40 species have been so far investigated. Wild non-human primates are difficult to spot and capture in the forest or savannah, therefore sampling is very difficult. Several approaches have been used to estimate SIVs prevalence in African nonhuman primate hosts, but all of them have limitations and therefore generate bias in prevalence estimates. NHPs living in zoos and in research centres are represented by individuals captured as juveniles or bred in captivity. It is known that

there is significant increase in SIV prevalence in adult, compared to juvenile wild-living monkeys [62, 128, 295], therefore results obtained from captive animals may not reflect the real SIV prevalence [223]. Moreover, non-natural close contact provides opportunities for cross-species transmission, as demonstrated by the examples of a chimpanzee infected in captivity in Cameroon [73], a white crowned mangabey in Kenya [375] and numerous macaques in the United States [125].

Wild-caught NHPs kept as pets have provided significant information concerning the diversity of SIVs [127, 232, 287]. Pet monkeys are captured when young, therefore the prevalence may be lower than that of a wild animal population, but may better reflect the SIV infection status in feral animals compared to captive ones. New representative SIV strains have been obtained by testing pet monkeys: SIVsmm in a sooty mangabey from Sierra Leone [62, 63], SIVrcm, a virus which naturally infects red-capped mangabeys [127] and six new SIV strains in NHP species from Cameroon [287]. The overall prevalence of SIVs was estimated to be 11.6% among the 215 pet monkeys sampled (55 adults and 160 between juveniles and infants) and infection rates varied from species to species [287].

To evaluate the magnitude of SIV exposure to humans, sampling of NHPs found at bushmeat markets have been set up. An extensive study measuring the overall SIV seroprevalence in 11 species sold in bushmeat markets ranged between 5 to 40% [287]. These results were within the same range of previous estimates of SIV prevalence in the wild. The major limitation of bushmeat sampling is that post-mortem samples could be in poor condition, thus often not allowing virus isolation and further biological characterization. Its main advantage is that it offers large numbers of samples in a short period of time. Additionally, this method could provide estimations of SIV prevalence in the wild, but it is unlikely to reflect prevalence of specific populations or social groups, since the meat which reaches the market could be brought from different regions or even be imported from neighbouring countries. Since bushmeat hunting is illegal in many countries, it is improbable that reliable information on its origin will be provided by the local traders.

In order to get a comprehensive and authentic picture of SIV prevalence in the wild, sampling NHPs in their natural habitat remains the best option to get a realistic

picture of the situation in the wild. Most NHPs are shy and considered highly endangered. Anaesthesia and blood collection are not without risk for these animals, therefore invasive procedures should be avoided in the field.

Recently, non-invasive diagnostic methods have been employed for serological analysis and viral RNA amplification in faeces and urine of wild chimpanzees [323] and captive sooty mangabeys [217]. Three additional studies making use of non-invasive sample collection methods investigated SIV prevalence in chimpanzee and gorilla populations inhabiting different regions of Cameroon [183, 384, 386]. However, despite sample discrimination, community or social group determination were not assessed because these primate populations were not habituated to the presence of human observers. Hence, the results obtained reflect a territory distribution of SIV more than the prevalence of SIV infection among social groups.

Although the collection of faecal samples has its obvious advantages, the nature of the samples precludes standardization, thus caution in results interpretation must be taken. For example, faeces from SIVmnd-1-infected mandrills in Central Gabon resulted so far to be negative, in spite of a very high prevalence of SIVmnd infection in that area (Clifford, personal communication cited by [8]). Moreover, wild-living SIV positive animals cannot be tracked, so the virus cannot be isolated nor can its *in vivo* pathogenesis be investigated.

A study conducted on a social group composed of a hundred habituated wild-living mangabeys from Taï National Park in Côte d'Ivoire, revealed a SIVsmm prevalence above 50% [322]. The advantage of tracking habituated animals resides in the opportunity to investigate SIV infection in all members of a social group and thus to better understand the SIV transmission patterns.

With these considerations in mind, I seized the opportunity to investigate the prevalence of SIV infection in different species of wild-living NHPs from West Africa. Several social groups which have been habituated to the presence of human observers were selected. This is clearly an advantage, but it did not eliminate all the difficulties compound with studying wild living animals. It surely offered a great opportunity to combine SIV results with host behavioural and ecological data in a natural setting.

In the next sub-chapter I situate the primate species studied for SIV infection in their taxonomic, geographic and ecological context.

## 1.7 Distribution, taxonomy, and socio-ecological characteristics of the non-human primate species investigated

In this thesis, nine diurnal primate species have been studied, although at a different extent. Three *colobus* species [*Colobus polykomos polykomos* (Zimmerman, 1780) - black and white colobus; *Piliocolobus badius badius and Piliocolobus badius temminckii* (Kerr, 1792) - western red colobus; *Procolobus verus* (van Beneden, 1838) - olive colobus], four *cercopithecus* species [*Cercopithecus diana diana* (Linnaeus, 1758) - Diana monkey; *Cercopithecus campbelli campbelli* – (Waterhouse, 1838) - Campbell's monkey; *Cercopithecus petaurista buettikoferi* (Jentink 1886) - lesser spot-nosed monkey; *Cercopithecus nictitans stampflii* (Jentink, 1888) - greater spot-nosed monkey] one *Cercocebus* species, *Cercocebus atys atys* (Audebert, 1797) - sooty mangabey and a pongidae species, *Pan troglodytes verus* - western chimpanzee (Schwarz, 1934).

Two field sites were selected: the Taï National Park, in Côte d'Ivoire and the Abuko Nature Reserve, in The Gambia, West Africa. The field sites are described in the next Chapter.

The NHP species we were interested in are found mainly in the western regions of sub-Saharan Africa (Figure 1.14).

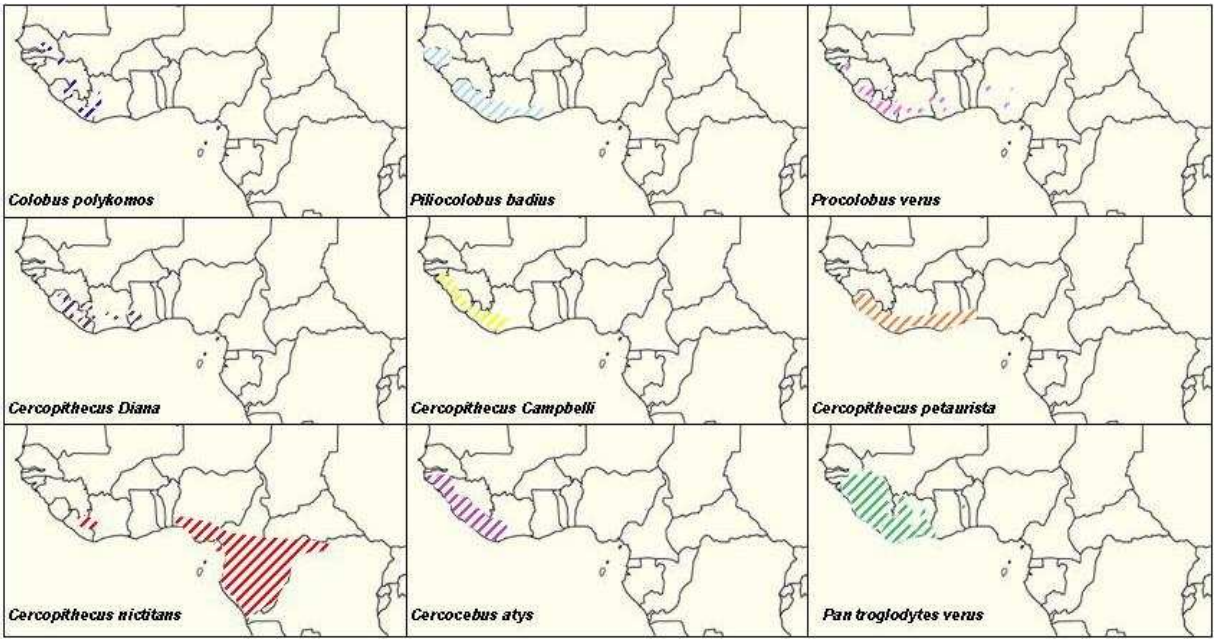



Figure 1.14: Geographical distribution of the primates species of interest. Adapted from [187]

Since I focused mainly on *Colobus* and *Cercopithecus* species in this study, I invite the reader to refer to other sources to get detailed information about *Cercocebus* and *Pan* species found in West Africa [32, 240].

*Colobus* and *Cercopithecus* species belong to the family of the Cercopithecidae, which is divided into 2 distinct sub-families: the Colobinae (leaf-eating monkeys) and Cercopithecinae (the cheeck-pouch monkeys). These extant subfamilies share distinctive morphological features, such as bilophodont molars, pronounced sexual dimorphism, and skeletal adaptations for rapid quadrupedal locomotion [86, 107]. They also differ in a number of respects. The colobines have diets that consist primarily (thought not exclusively) of leaves of trees and shrubs. Along with this specialised diet, these leaf-eating monkeys have ruminant stomachs and a dental morphology suited for shearing leaves [86]. Colobines are almost exclusively arboreal and posses a reduced or absent pollux and long nonprehensile tails [107]. In contrast, cercopithecines have well developed thumbs and tails of varying lengths. These monkeys are omnivorous, concentrating on fruit and possess sacs in the buccal region of the oral cavity called

cheek-pouches, which are used to store food. The Cercopithecines are not exclusively arboreal and are morphologically diverse [100].

Oates provides an excellent description of the African colobines [272]. Several anatomical differences exist between *Piliocolobus*, *Procolobus* and *Colobus*. Only the females of *Piliocolobus* and *Procolobus* develop sexual swellings. Males of *Pilio*- and *Procolobus* show distinct ischial callosities, contrary to the joint callosities in *Colobus* males. *Colobus* has a three lobed stomach, whereas *Pilio*- and *Procolobus* have a sacculated stomach divided in four chambers. *Colobus* has a sub-hyoïdian sac (absent in the other two) and a big larynx, which allows for low frequency loud calls.

Among the colobines, we find all possible dispersal patterns: male-biased dispersal in *C. polykomos*, female-biased dispersal in *P. badius* and dispersal by both sexes in *P. verus*. All guenons and the sooty mangabeys have male-biased dispersal. The degree of bias, the frequency of dispersal and dispersal distances remain unresolved mysteries, however. The mating systems of the colobines are equally variable: a harem system in *C. polykomos*, multi-male groups in *P. badius*, and small groups with variable membership in *P. verus*. All guenons have harem systems, while the sooty mangabeys live in extremely large multi-male groups.

*Colobus* and *Cercopithecus* monkeys living in the Taï Forest are preyed upon by leopards [161, 427, 428], crowned-hawk eagles [428], chimpanzees [31, 41, 259, 428] and humans. Despite the common predators, these species have entirely different anti-predation strategies. The red colobus lives in large groups, frequently forms poly-specific associations and male coalitions attack predators [31, 33, 41, 259].The black-and-white colobus lives in intermediately sized groups and is cryptic [84, 191, 193, 228]. It does not associate in poly-specific groups more than expected by chance and the adult male may actively deter predators through attacks and loud calls [240]. The olive colobus lives in a permanent poly-specific association with a particular partner Diana monkey group (in other areas sometimes with other guenon species) and leads a very cryptic life [191, 269, 274]. In Abuko Nature Reserve*, Piliocolobus badius temminckii* live sympatrically with patas and vervet monkeys (*Erythrocebus patas* and *Cercopithecus aethiops sabaeus*), but are not influenced by any predator pressure [354, 355].

Polyspecific associations are relatively widespread among the guenons, and benefits resulting from such associations fall into two broad categories: resource acquisition and predator avoidance [100]. Further details on socio-ecological characteristics of the colobus and guenon species investigated are also presented in Table 1.2 and in chapters 6, 9 and 10.

In general, it is important to consider host behavioural-ecology data because these parameters will influence disease risk. More specifically, the risk of acquiring SIV (or other sexually transmitted diseases) should be higher in animals with promiscuous mating systems (Loehle 1995; Lockardt et al 1996; Heymann 1999; Nunn et al. 2000) or in populations with higher variance in male mating success (Thrall et al. 2000). The latter effect arises because a few individuals with large numbers of mating partners can serve as ‘super spreaders’ for infections to spread through populations. Given that SIV can potentially be transmitted also vertically or via aggressive contact between individuals, other parameters than mating system and reproduction need to be considered. Host density and group size, individual differences in dominance rank, migration, diet, habitat use and environmental factors, including habitat characteristics and seasonality, are all parameters that would shape the structure of a social group and the interactions and competitions for mates and food resources between and within the social groups. These aspects will be discussed in more details in Chapter 9.

Table 1.2: General socio-ecological data on NHP species of interest living in Taï National Park.

Source: [32, 40, 50, 86, 98, 119, 191, 194, 195, 240, 306, 354]

| | ***Piliocolobus badius spp.*** | ***Colobus polykomos polykomos*** | ***Procolobus verus*** | ***Cercopithecus diana diana*** | ***Cercopithecus campbelli*** | ***Cercopithecus petaurista*** | ***Cercopithecus nictitans*** | ***Cercocebus atys atys*** | ***Pan troglodytes verus*** |
|---|---|---|---|---|---|---|---|---|---|
| **Sexual Dimorphism** | little (♂body weight: 8.3 kg; ♀: 8.2 kg) | yes (♂body weight: 9.9 kg; ♀: 8.3 kg) | little (♂body weight: 4.7 kg; ♀: 4.2 kg) | yes (♂body weight: 5.2 kg; ♀: 3.9 kg) | yes (♂body weight: 4.5 kg; ♀: 2.7 kg) | yes (♂body weight: 4.4 kg; ♀: 2.9 kg) | yes (♂body weight: 6.4 kg; ♀: 4.1 kg) | yes (♂body weight: 11 kg; ♀: 6.2 kg) | yes (♂body weight: 40-60 kg; ♀: 32-47 kg) |
| **Sexual swelling** | yes | no | yes | no | no | no | no | yes | yes |
| **Group size** | 40-90 individuals | 1-2 adult ♂ 3-7 adult ♀ 6-12 infants, juveniles, infants | several adult♂ three or more adult ♀ and infants | single adult ♂, 6 or 7 ♀ and their offspring | single adult ♂, 3 or 4 ♀ and their offspring | single adult ♂, 4 or 8 ♀ and their offspring | single adult ♂, 4 ♀ and their offspring | around 100 individuals | form 10 up to 100 individuals |
| **Group composition** | multi-male/ multi-female | one-two males | multi-male/ multi-female | single male | single male | single male | single male | multi-male/ multi-female | multi-male/ multi-female |
| **Group transfer** | male phylopatry | female phylopatry | both sexes disperse | female phylopatry | female phylopatry | female phylopatry | female phylopatry | female phylopatry | male phylopatry |
| **Polyspecific association** | yes | tends to avoid associating with sympatric species | yes, especially with diana monkeys | yes, especially with olive colobus | yes, with other *Cercopithecus* species, especially *C. petaurista* | yes, with other *Cercopithecus* species | no (in Taï) | yes, mostly with diana monkeys | no |
| **Layers of forest used** | all, but prefer main canopy | all | forest understory | all | forest understory and also ground level | lower level of forest | all, but prefer high canopy | predominantly terrestrial | arboreal and terrestrial |
| **Diet** | leaves, fruits, flowers | marked preference for fruit seeds and liana leaves | most preferred are young leaves with fruit | large amounts of fruit and insects with smaller amount of flowers and leaves | fruit and insects, flowers and leaves | fruit, flowers, Insects | fruit, leaves, insects | fruit, insects, leaves | fruit, leaves, flowers, seeds, animal prey |
| **IUCN list** | endangered | near threatened | near threatened | endangered | not threatened | not threatened | critically endangered | near threatened | endangered |

## 1.8 Poaching pressure, bushmeat trade and consumption in Africa

One of the widely accepted hypothesis today is that SIVcpz and SIVsm have been transmitted to humans as a result of cutaneous or mucous membrane exposure to infected animal blood during bushmeat hunting or food preparation or as a result of scratches or bites inflicted by NHPs kept as pets [143]. These practices and customs constitute risk factors for the emergence of novel diseases in the human population. They are continuously expanding, thus representing an increasing threat for cross-species pathogen transmission. Monitoring the presence of pathogens, including SIV in frequently captured NPHs is therefore one of the key factors to measure the likelihood of emerging disease outbreaks.

Many case studies across West-equatorial Africa report bushmeat hunting as a major source of food for many local communities. Primates are often the target of such hunting activities [103, 106, 231]. The relatively high density of human population in West Africa has already resulted in local extinctions throughout the Upper Guinea Forest Ecosystem [45].Hunting has been identified as the primary cause in the near extinction of Miss Waldron's red colobus (Procolobus badius waldroni) in West Africa [270], of which only a few remnant populations remain [236].

Côte d'Ivoire and the Taï National Park (TNP) are not spared from these activities. The monthly reports of the *Division Aménagement du Parc National Taï* (PACPNT) state that the diversity of game meat seized during several anti-poaching controls in TNP (1993 - 1997) is dominated by monkeys (nearly 40 %), followed by all species of the duiker group (more than 35 %) [280]. The TNP study area is located in the less developed region of Côte d'Ivoire's forest zone. Population density is below the average, the timber industry and agriculture are poorly developed and islands of natural forest do still exist. Species diversity within the group of mammals exploited by subsistence hunters is still high, even outside the protected areas. In contrast to degraded forest regions, where the range of hunted species is dominated by the greater cane rat, the African brush-tailed porcupine, the blue duiker and the bushbuck, hunters in the south-western region trap and shoot all species of primates (including the chimpanzee) and forest antelopes (including the Zebra Antelope and the Jentink's

duiker) [54]. We know that certain areas of Taï National Park have come under heavy pressure from new settlers. Since then, timber exploitation has opened up large areas neighbouring the park for agriculture, and farmers from the Sahel region have immigrated into the area. In 8 years, the human population density has increased sixfold leading to encroachment and slash-and burn activities in the park [57]. Caspary conducted a study on wildlife utilization in Côte d'Ivoire, and found that the vast majority (90%) of hunters are farmers who hunt for both subsistence and commercial purposes. Rural hunters are linked with urban-based merchants and restaurant owners in a well-organized, but illicit, commerce in wild game meat. Despite a ban on hunting that dates from the early 1970s, the economic value of bushmeat amounted to 1.4% of Côte d'Ivoire's gross domestic product (GDP) in 1996 [53].

In central Africa only, it is estimated that 1 million metric tons of wildlife is consumed every year [407]. The Congo Basin is the biggest centre for bushmeat hunting worldwide and in many areas bushmeat makes up 80% of people's protein intake [285]. The local demand for and consumption of bushmeat is high, [200] and the extent and intensity of commercial hunting has increased dramatically over the past decade [11, 36, 409].

Factors underlying the increase of bushmeat demand include human population growth, the absence of effective alternatives to hunting for meat, appealing short-term economic benefits from the commercial bushmeat trade, lack of capacity to enforce national and international legislation, logging which allows hunters to penetrate deeper into formerly untouched forests, oil industry which also induces large influxes of people into tropical forests, insufficient national and international awareness, and a lack of willingness to take action [45]. Wars also have a deleterious impact on the environment, as armed conflicts facilitate bushmeat trade and consumption. In Central Africa, for example, the greater availability of firearms associated with warfare makes large mammals easier to hunt and sell, while human populations displaced by hostilities are more dependent on consuming and trading in natural resources after their preconflict livelihoods have been destroyed [96, 97]. In addition, the collapse of formal state institutions, whose mandates are to regulate the unsustainable use of natural resources

[299], allows actors in the government, military, and private sector to use violence to gain unrestricted access to economically valuable natural resources [204, 381].

# 2. Aims and objectives

The **aims** of this study were (i) to investigate with non-invasive methods the presence and the prevalence of SIV in several non-human primate species living in Taï National Park, Côte d'Ivoire (ii) to explore and compare SIV infection in different primate subspecies living in geographically distinct regions to further document and characterise SIVs in West Africa, and (iii) to better estimate the likelihood of these SIVs to cross the species barrier toward the West African chimpanzee species and ultimately toward the human population.

**Objective 1:** To identify suitable markers for the isolation of microsatellite loci in seven primate species, thus enabling discrimination of faecal samples collected

**Objective 2:** To assess the presence of SIV infection in several primate species inhabiting the Taï Forest and to determine the prevalence of infection

**Objective 3:** To compare SIV phylogenetic results with host group distribution on a micro- and macro-scale by integrating long term behavioural and ecological data

**Objective 4:** To assess the presence of SIV infection in a red colobus subspecies living in The Gambia and to molecularly compare this virus with that present in another subspecies of red colobus living in Côte d'Ivoire

**Objective 5:** To fully characterise the SIV genomes of two colobus species from Côte d'Ivoire

# 3. PhD history and collaborations

The initial PhD proposal, drafted at the end of 2003, represented an essential component of a multidisciplinary project entitled: “The risk of infection by novel HIV-varieties in human populations in direct contact with free-living African monkeys.” The overall general purpose was to investigate which factors determine the risk of emergence of a novel viral infection in the human population originating from cross-species transmissions of simian immunodeficiency viruses (SIVs) carried by non-human primates. We assumed that these risks were directly related to the frequency of contact between people and non-human primates (hunting and handling infected animals), the genetic diversity of SIVs they harbour, and the presence of strains capable of infecting multiple species of non-human primates. The Taï National Park in Côte d’Ivoire offered an ideal platform for this investigation. Long-term ecologic and behavioural studies have been conducted there since 1992 and several groups of non-human primates including black and white, red and olive colobus, several *Cercopithecus* species, the sooty mangabey and the chimpanzee have been habituated to the presence of researchers.

Three main axis of research **were** created to tackle this topic:

1. The behaviour-ecology axis, directed by Prof. Ronald Noë, Senior lecturer, Faculty of Science, University of Basel, and Ethology of Primates, Univ. Louis-Pasteur, Strasbourg, France and Prof. Peter Nagel, Institute of Environmental Sciences (NLU) - Biogeography, Basel University
2. The host population genetics axis, led by Prof. Michael W. Bruford, Cardiff School of Biosciences, Cardiff University, UK and Dr. Roland Molenda, head of the Molecular Ecology Laboratory in Basel
3. The virology axis directed by Dr. Martine Peeters, Retrovirus Laboratory, IRD, Montpellier, France and Prof. Georg Pauli, Robert Koch Institute, Berlin, Germany

Prof. Marcel Tanner, Director of the Swiss Tropical Institute in Basel and Head of the 'Centre Suisse de Recherche Scientifique' (CRSC) in Abidjan, Côte d'Ivoire, Dr. Johannes Refisch, co-director of the Taï Monkey Project and Dr. Jakob Zinnstag, working on the Human-animal disease interface at the STI were also involved in the early phase of the project.

A pilot study of 4 to 6 months, sponsored by the National Geographic foundation and the Commission for Research Partnerships with Developing Countries (KFPE) was set up to assess the project feasibility on different levels. Three students, including myself, participated in the initial field phase of this project. The objectives were to collect faecal samples from habituated groups of *Colobus* and *Cercopithecus* monkeys, to proceed with the recognition of individuals of different *Colobus* groups, to gather preliminary data on density and distribution of habituated monkey populations and to estimate the feasibility of collecting additional data on non-habituated groups in areas of the park exposed to a different anthropogenic pressure. This pilot study was also aimed at evaluating the feasibility of collecting data on the social structure of the *Colobus* monkeys, and their association rates with other monkey species. The host and viral genetic information potentially obtainable by extracting DNA and RNA from collected faecal samples, together with behavioural and ecological data on the different monkey groups, would have allowed to cross-check and to validate the genetic results on relatedness, paternity and reproductive success, thus contributing to the understanding of the behavioural basis of the genetic structure. Moreover, thorough data on anthropogenic (mainly poaching) pressure would have allowed assessment of the risks to which humans are exposed when hunting and handling non-human primates.

The political instability in Côte d'Ivoire and the lack of major financial support prevented this team from pursuing every intended aspect of this project. My two student colleagues were redirected to other topics of research. Following completion of the pilot sample collection, I received financial support from a private Swiss foundation to investigate monkey population genetics in the laboratory of Prof M. W. Bruford at the School of Bioscience in Cardiff, UK.

Work conducted in his lab concentrates on improving the understanding of primate societies and how behaviour and reproduction correlate with social and genetic

structure in primate species as diverse as the grey mouse lemur, Hanuman langur, Bornean orang-utan and the western lowland gorilla, as well as other mammals such as the black rhinoceros. Common among most of these projects has been the need to use non-invasively collected material - faeces and hair. Over the last three years his laboratory has developed a number of methods to efficiently extract DNA and genotype such samples. With the help of Dr. A. Roeder, I started to screen human markers suitable for cross-species amplification of Old World monkeys' microsatellite loci. Subsequently, I optimised the experimental conditions to obtain replicable and accurate results. In six months I was able to analyse the core of the dataset, which consisted of samples from known members of habituated study groups, but which included also unknown individuals. Nine suitable loci were isolated, but to complete the genotyping, we applied for an additional grant. When funding was not obtained, I sought an opportunity to work with Dr. Martine Peeters to screen the samples collected in Côte d'Ivoire for SIV at the Institut de Recherche pour le Dévéloppement (IRD), in Montpellier.

Dr. Martine Peeters is a virologist who is responsible for conducting research involving 1. The dynamics of the viral sub-types implicated in the HIV pandemics in Africa and the characteristics of the circulating viruses. 2. The consequences of the genetic diversity of HIV. 3. The antiretroviral drugs efficacy on HIV African strains and on evolution of treatment. 4. The surveillance of a potential emergence of novel variants of HIV, following cross-species transmissions with the simian counterpart SIV, and the prevalence and characterisation of SIVs from primates inhabiting the equatorial forests of Central Africa.

In the late nineties, this laboratory established a long-term collaboration with research partners in Cameroon. This collaboration allowed the collection and the analysis of thousands of tissue samples from monkeys found at bushmeat markets, which led to the characterisation of six new SIVs. Serological diagnostic tests were then developed to screen additional simian samples, but also to weight the risk of emergence of new HIVs, closely related to these newly identified SIVs, in the human population. This laboratory also focused on the prevalence and diversity of SIVcpz in wild chimpanzees. In collaboration with Dr. Beatrice Hahn from the University of

Birmingham, Alabama, a non-invasive technique was developed, allowing SIV antibody detection by cross-reaction with HIV antigens and the isolation of viral RNA from faecal samples. An extensive faecal sample collection of chimpanzees and lowland gorillas living in the tropical forests of Cameroon substantiated the hypothesis of the origin of HIV-1 in these regions of Africa. This project continues today, and aims at further identifying and characterising new SIVs and at determining their prevalence in wild-living simian populations. Investigating the presence of SIV in *Colobus* species, whose range encompasses many regions of equatorial Africa, represented another topic of interest for the research team. The provision of samples collected in West Africa during my pilot study, together with behavioural and ecological information on this species would have contributed to a better understanding of the origin and evolution of SIV.

With this aim in mind, I entered collaboration with Nelson Ting, a graduate student from the Anthropology Department of the City University of New York. Mr. Ting is interested in studying mitochondrial relationships and divergence dates of the African colobines. He kindly sent us a subset of faecal and tissue samples of a different subspecies of western red colobus from The Gambia to compare with those of Côte d'Ivoire.

Finally, after having finished my field and laboratory work, I completed my PhD program at the Swiss Tropical Institute in Basel, where I attended classes in the frame of the course on Public Health and Epidemiology.

# 4. Study sites, primate species and methods

## 4.1 Study sites

Two West African study sites have been selected for this thesis. The first is Taï National Park (TNP), in Côte d'Ivoire and the second is Abuko Nature Reserve in The Gambia (Figure 4.1).

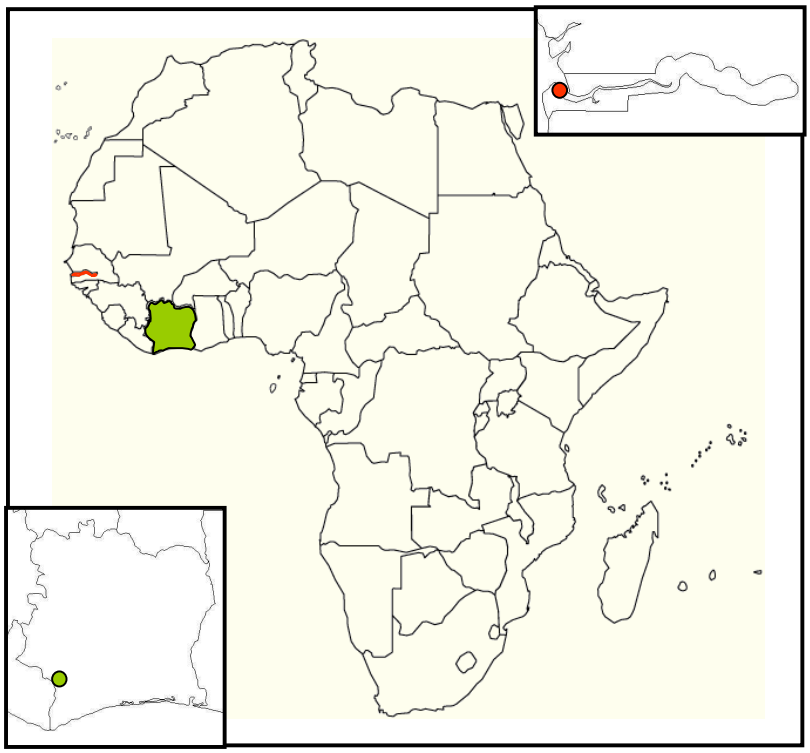


Figure 4.1: Taï National Park - Côte d'Ivoire (in green) and Abuko Nature Reserve - The Gambia (in red)

### 4.1.1 The Taï National Park, Côte d'Ivoire

The Taï National Park is the last substantial block of intact forest remaining in West Africa. It is located in the southwest corner of Côte d'Ivoire, near the Liberian border, about 200 km south of Man and 100 km from the Gulf of Guinea coast in the districts of Guiglo and Sassandra (0°15'-6°07'N, 7° 25'-7°54'W). The forest was once

part of a large forest belt in the Upper Guinea Forest that covered a vast area from Ghana to Sierra Leone. Its decline in size has been dramatic, particularly in the twentieth century [230]. The Park was recognised as such in 1972 and was declared a UNESCO World Heritage Site in 1982. The official area today is 330,000 ha in addition to a 20,000 ha buffer zone, which is contiguous to the 73,000 ha «Réserve de Faune du N'Zo» to the north of the park (Figure 4.2) [54].

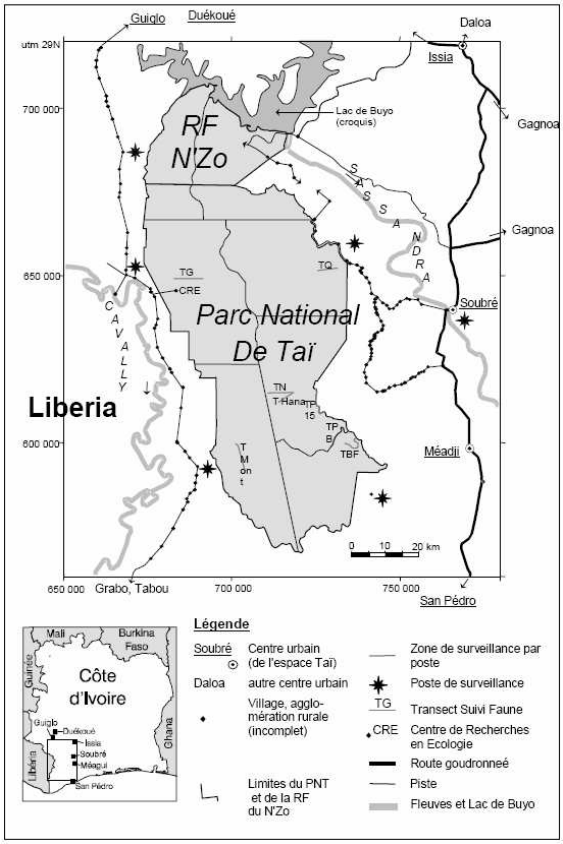



Figure 4.2: Location of TNP in south-western Côte-d'Ivoire

There are two distinct climatic zones with annual average rainfall of 1,700 mm in the north and 2,200 mm in the south. The rains peak in June and September and there is a marked dry season from December to February. Temperatures range from 24℃ to 27℃ and the relative humidity is constantly high, between 85 and 90%.

Altitudes range from 80 m to 396 m, with Mount Niénokoué as the highest peak. The soils are ferralitic and of generally low fertility. About 88% of the Taï National Park surface is drained by different tributaries of the Cavally river, notably by the Audrénisrou,

the Hana, the Nzé and the Meno. The park contains some 1,300 species of higher plants including 150 identified as endemic. The vegetation is predominantly dense evergreen ombrophilus forest of a Guinean type with a 40-60 m high continuous canopy and large numbers of epiphytes and lianas. The forest is recovering from commercial timber exploitation, which officially ceased in 1972. The park contains a fauna typical of West African forests. The forest is inhabited by 12 primate species, three of which are nocturnal. Almost 1,000 species of vertebrates including over 230 bird species have been identified in the park [239].

Since Côte d'Ivoire began to attract people, because of the prospect of easily grown cash crop like coffee and cocoa, and more recently oil-palm and hevea trees, the Taï National Park has become an island within a rapidly growing agricultural landscape. The human population around the park increased from 23 000 in 1956 to 375 000 in 1988. In 1998, this number has been estimated to reach 527 000 people [54]. The human density increased in the Taï sous-préfecture, close to the study site, from 8 inhabitants per $km^2$ in 1971 to 135 inhabitants per $km^2$ in 1991. In addition, the civil war that started in Liberia around 1990 produced a dramatic flow of refugees into the bordering area of Côte d'Ivoire. This resulted in a further population increase of 400% in the five villages near the Taï Chimpanzee Project (TCP) and the Taï Monkey Project (TMP) research stations (19 347 refugees for 6337 residents). In 1997 the refugees started slowly to move back to Liberia, but this demographic pressure reduced dramatically the surface of the forest in this region and poaching for meat increased considerably around and within the park. Critical living conditions during the past five years of political crisis increased further the pressure on the park natural resources.

### 4.1.2 The Taï Monkey Project

The Taï Monkey project was founded in 1989 when Ronald Noë and Bettie Sluijter undertook a pilot study on red colobus monkeys. In 1991 Klaus Zuberbühler and Kathy Holenweg started to habituate the first group of red colobus and Diana monkeys and established the primary study grid. They selected an area with a high density of monkeys near the field station of the « Institut d'Ecologie Tropicale » (IET) on the western border of the park. The IET research station is located at approximately 20km

from the nearest village and 25 km from the Cavally river that forms the border with Liberia. The grid established in 1991 has since been enlarged, but still forms the core of the project study site, located between 6°20 N to 5°10 N and 4°20 ´W to 6°50 W. The study site has been described in detail previously [32, 39, 235, 428].

By the end of 1994, there were - on average - six students and six field assistants studying monkeys at anyone time. The breadth of research topics has increased over the years going from anti-predator association, feeding, ranging, vocal and social behaviour to conservation and positional behaviour. More details on the research conducted in the Taï monkey Project are found in “Monkeys of the Taï Forest : an African primate community” [240].

### 4.1.3 The Abuko Nature Reserve, The Gambia

The Abuko Nature Reserve was the Gambia’s first officially declared reserve in1968. It is located in the western division of the country (13.41℃N, 16.65℃W). The 106.6 ha reserve is a mosaic habitat consisting mostly of tree and shrub savannah (56.6%), woodland savannah (24.8%), and gallery forest (16%). It is situated at an altitude of 5.5-16 m and the average annual rainfall is 1181 mm. The daily minimum temperature ranges between 19.4° and 20.4℃ and the daily maximum between 28.1° and 32℃. The rectangular-shaped reserve is completely surrounded by human habitation, fallow and or cultivated fields [355].

The Gambia hosts more than 550 bird species and at least 99 species of mammals have been identified. It has been estimated that the country harbours 974 species of vascular plants. Four primate species occur in the reserve. The Gambia lays at the northern limit of the western chimpanzee subspecies distribution [52].

## 4.2 Primate species and distribution

### 4.2.1 Primate species and social groups selected – Taï National Park

Picture 4.1: The nine diurnal NHP species found in Taï National Park
1. *Piliocolobus badius*; 2. *Cercopithecus diana*; 3. *Cercocebus atys*; 4. *Colobus polykomos*; 5. *Cercopithecus Campbelli*; 6. *Pan troglodytes verus*; 7. *Procolobus verus*; 8. *Cercopithecus petaurista*; 9. *Cercopithecus nictitans.* Pictures 1, 3, 4, 6: S. Locatelli; 2, 5, 7: F. Moellers; 8, 9: N. Rowe.

Two social groups of western red colobus (*Piliocolobus badius badius*) (Bad1b, Bad2a), two of black and white colobus (*Colobus polykomos*) (Pol1, Pol3), three of olive colobus (*Procolobus verus*) (Ver1, Ver2, Ver3), three of Diana monkeys (*Cercopithecus diana*) (Dia1, Dia2, Dia3), one group of Campbell's monkeys (*Cercopithecus Campbelli*), one of lesser spot-nosed monkeys (*Cercopithecus petaurista*) and one of greater spot-nosed monkeys (*Cercopithecus nictitans*) were selected for faecal samples collection (Picture 4.1). We also collected samples from a social group of sooty mangabeys, which

were sent to Yerkes Primate Centre for analysis. All the above mentioned groups occur in the 3.5 by 2.5 km grid system, except the greater spot-nosed monkey group. These social groups have been habituated to the presence of researchers since the 1990's (Figure 4.3). Their range and association with other monkey species has been previously determined and will be discussed in the next chapters. Many individuals were recognised by the observers who were collecting data. Recognition of monkeys was taught to new researchers by previous researcher or by field assistants. To date, four social groups of chimpanzees have been habituated to the presence of researchers, but live outside of this grid system, although one chimpanzees' group range (middle group) covers this area as well. For more details on the chimpanzees communities please refer to 'The chimpanzees of the Taï Forest [32]. No faecal samples from chimpanzees were collected.

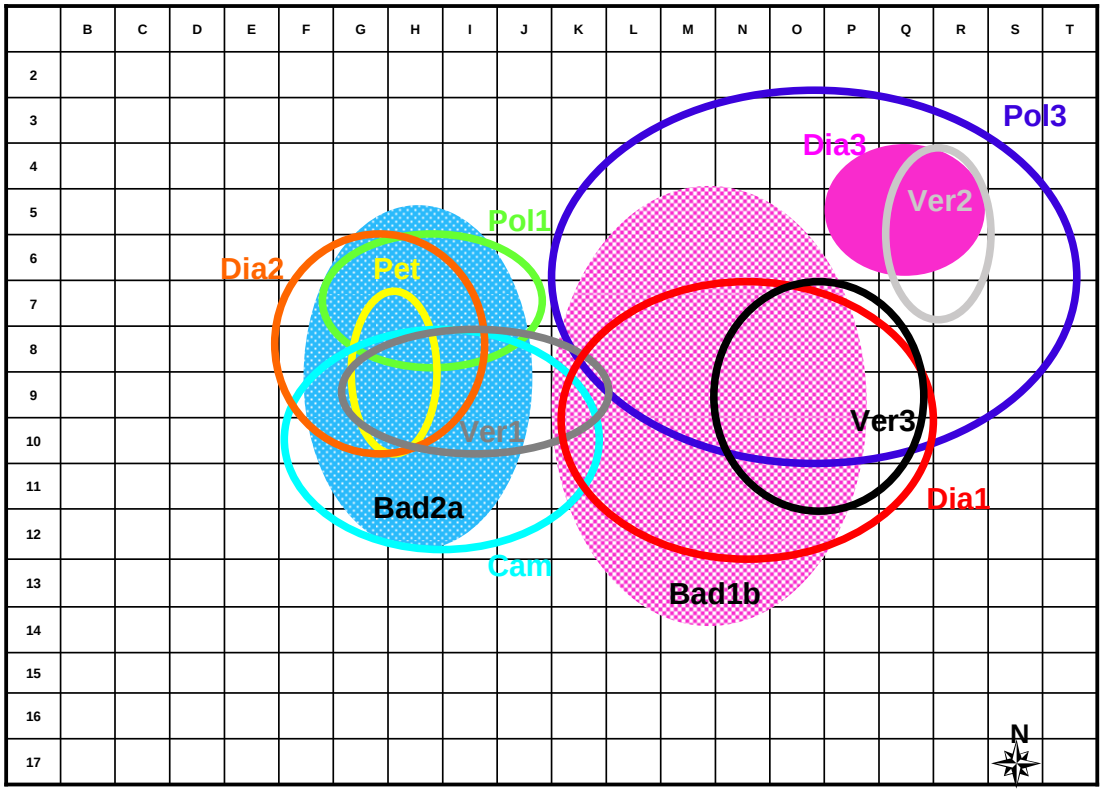



Figure 4.3: Distribution of the NHP groups under study occurring in the 3.5 by 2.5 km study grid. Two social groups of western red colobus (Bad1b, Bad2a), two of black and white colobus (Pol1, Pol3), three of olive colobus (Ver1, Ver2, Ver3), three of Diana monkeys (Dia1, Dia2, Dia3), one group of Campbell's monkeys (Cam) and one of lesser spot-nosed monkeys (Pet) are represented.

### 4.2.2 Primate species and social groups selected – Abuko Nature Reserve

Three diurnal primate species, the Temminck's red colobus (*Piliocolobus badius temminckii*), the patas monkey (*Erythrocebus patas)*, the vervet/green monkey (*Cercopithecus aethiops sabaeus*) and a nocturnal primate, the bushbaby (*Galago senegalensis*) live in Abuko Nature reserve (Picture 4.2). The most documented primate species in the reserve is Temminck's red colobus. The Abuko Nature Reserve contains five troops of Temminck's red colobus with the largest troop containing less than 35 individuals (average density 123.7 colobus per $km^2$) [354].

Picture 4.2: The three diurnal NHP species found in Abuko Nature reserve

(*Piliocolobus badius temminckii* - Temminck's red colobus; *Erythrocebus patas* - patas monkey; *Cercopithecus aethiops sabaeus*- vervet/green monkey) (www.photosearch.com)

## 4.3 Methods

In this study we focused on habituated non-human primate social groups. Habituated groups are a precious commodity. Large scale invasive sampling can jeopardise the habituation efforts accomplished over many years and could also potentially affect the life of the individuals sampled. Therefore, instead of acquiring information from blood or tissue samples (except for rare cases, when carcasses were found on the forest floor) we adopted a non-invasive approach and we collected freshly dropped faecal samples from known species, social groups and possibly, visually identified individuals. Host DNA, antibodies and viral fragments can be extracted from faecal matter. Genetic material can be isolated from cells shed from the intestinal epithelium, allowing amplification, sequence analysis and evolutionary study of host and viral genomes.

In the next few pages I will give a brief overview of the methods applied in this thesis. Detailed description of the laboratory procedures are given in the material and method sections of the following research chapters.

### 4.3.1 Species and individuals identification

In the Taï Forest, on most observation days, monkey groups were followed from dawn to dusk. The territorial range of every social group was known from previous studies, and for some groups, individuals were identified. Associations between groups of different species (polyspecific associations) were known and were stable. Their location was facilitated by the groups' vocalisations and loud alarm calls.

Group membership of red colobus groups living in Abuko Nature Reserve was difficult to determine, since they perform fission-fusion behaviour there (Nelson Ting, personal communication). Faecal samples were collected first thing in the morning underneath the sleeping trees, from opposite ends of the radius of the red colobus range, to ensure sampling from different individuals.

### 4.3.2 Faecal sample collection and storage

Faeces were collected using a stick or a sterile plastic or wooden spoon (to avoid contamination with the collector's DNA), immediately after defecation, to approximately half-fill a clean, possibly sterile 15 ml collection tube, on which relevant information (territory coordinates, identity, sex and age of the individual, when known, as well as date, name of the collector and time of collection) were noted. Disposable gloves were worn and faeces were never touched with bare hands to avoid cross-contamination of the samples and potential disease transmission. The rest of the tube was filled with storage solution. There are different preservation methods: some of the best methods to preserve faeces are silica gel beads to dry the sample, a DMSO-EDTA-tris-salt solution or 70% / absolute ethanol, stored at -20°C or in liquid nitrogen. We used RNA*later*® (Ambion, Inc.) which is probably the optimal method. Expensive, but excellent for the preservation not only of DNA, but also RNA. RNA*later*® is an aqueous, non toxic storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA *in situ* in unfrozen specimens. RNA*later*® eliminates the need to immediately process

specimens or to freeze samples in liquid nitrogen for later processing, extremely advantageous features when field sites are situated in remote areas away from laboratory facilities. In the field, samples were stored at 4℃. After shipment at room temperature, samples were put at -80℃ for archival storage (Figure 4.4).

Figure 4.4: Schematic drawing featuring NHPs observation, faecal samples detection, collection and storage

### 4.3.3 Faecal/tissue DNA extraction

We chose QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), an extraction method eliminating many compounds that can degrade DNA and inhibit downstream enzymatic reactions. The purified DNA, free of protein, nucleases, and other impurities or inhibitors is then ready for use in Polymerase Chain Reactions (PCR) and other enzymatic reactions, and can be stored at – 20℃ fo r later use.

### 4.3.4 Microsatellites loci selection and amplification

For genetic investigations, individuals are characterised at a number of highly polymorphic microsatellite loci. These loci are genomic segments that contain tandemly repeated units (1 to 6 nucleotides long) flanked by non repetitive sequences. By amplifying a locus using the PCR, with primers placed in the segments flanking the repeats of interest, one can characterise individual variation. In this study, microsatellite analysis was performed as a supporting tool to discriminate faecal samples collected from individuals belonging to the same social group.

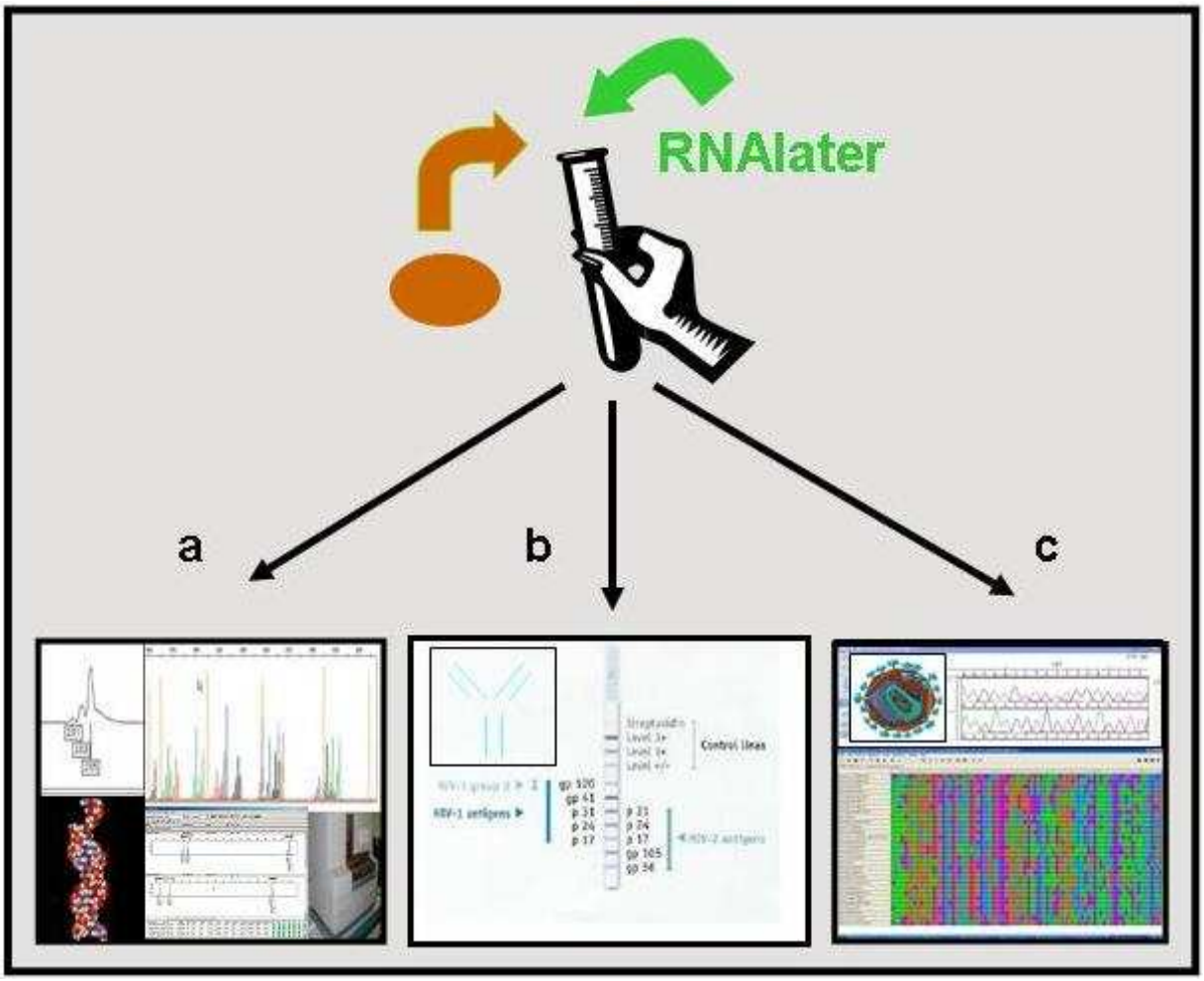



Figure 4.5: DNA extraction and microsatellite analysis (a), antibody extraction and HIV cross-reactive antibody test (INNO-LIA) (b), RNA extraction, RT-PCR and sequencing (c) from a single faecal sample conserved in RNA*later*

### 4.3.5 Multiplex Polymerase Chain Reaction and fragment analysis

Multiplex PCR is a powerful technique that enables amplification of two or more microsatellite loci in parallel in a single reaction tube. It is particularly suitable when genotyping implies the analysis of many loci, but when little DNA is available.

Amplification products were separated using capillary electrophoresis (ABI 3100 - Applied Biosystems, UK). Alleles were sized relative to an internal size standard using Gene Scan 3.7 (Perkin-Elmer Applied Biosystems). Alleles frequencies and expected heterozygosity were calculated using Genepop v 3.4 online [308].

### 4.3.6 Detection of HIV-1 cross-reactive antibodies in faecal samples

There aren't yet any sensitive and simultaneously specific assays, which would allow for screening SIV antibodies in faecal samples of non-human primates. Hence, we analysed the samples using commercial HIV confirmatory assays. The rationale lies in the idea that SIV antibodies may cross-react with HIV-1 or HIV-2 antigens and therefore, this assay may be used for initial screening of SIV positive samples. Two types of assays are available: western blots and Line Immuno Assays. The INNO-LIA™ HIV I/II Score is a Line Immuno Assay (LIA®) (Innogenetics, Ghent, Belgium). It is used to confirm the presence of antibodies against the human immunodeficiency virus type 1 (HIV-1), including group O, and type 2 (HIV-2) in human serum or plasma. This test has also been used in the past to successfully identify SIV in blood samples of various primate species as well as in faecal samples from chimpanzees and gorillas. This assay needed first of all to be adapted for testing faecal samples conserved in RNA*later*™, a storage solution with a high content of salt. RNA*later*™ precipitates immunoglobulines, precluding antibody detection. This problem can be circumvented by diluting faecal/RNA*later*™ mixtures with PBS-Tween-20, by heat - inactivating the mixture and then by clarifying the samples by centrifugation. At this point the sample can be dialyzed against PBS for two consecutive nights at 4ºC and it will then be ready for serological testing.

### 4.3.7 Faecal viral RNA extraction

To extract RNA from discarded intestinal endothelium cells from faecal matter, we used RNAqueuous-Midi™ (Ambion, Austin, Texas, USA), which is a phenol-free, glass fiber filter-based procedure for cellular RNA isolation. The RNA recovered is usually at a suitable concentration to be used directly for RT-PCR and most other common applications.

### 4.3.8 Amplification of SIV sequences from faecal RNA

In order to obtain cDNA from faecal viral RNA, we performed reverse trancriptase polymerase chain reaction (RT-PCR) using universal as well as consensus primers targeting different regions of the SIV genome. cDNA was synthesised using the outer reverse (R1) primer, followed by a nested PCR using primers F1/R1 in the first round and F2/R2 in the second round (see following chapters for specific details). We used the Expand™ Reverse trancriptase for reverse transcriptase reaction and the Expand™ Long Template PCR system (Roche Diagnostic, Indianapolis, IN) for nested PCR, according to the manufacturer's instructions.

**Amplification of full-length viral sequences from tissue extracted DNA**

A complete genome was amplified by utilising a combination of consensus and virus specific primers targeting a series of overlapping fragments described in details in chapters 7 and 8.

**Sequencing and sequences assembling**

The resulting amplification products were purified on agarose gel (Quiagen, Valencia, CA) and directly sequenced by the "Dye-terminator sequencing method", with the Big Dye Terminator™ kit v 3.1 following the manufacturer's instructions. Sequences were obtained using an automated sequencer (3130xl Genetic Analyser, Applied Biosystem, Courtaboeuf, France). Control of the fragments and assembly of the sequence fragments to a full-length genome were performed with SeqMan II (DNASTAR, Madison, WI). Sequences alignments for further phylogenetic analysis were performed using ClustalW (freely available on the internet).

### 4.3.9 Statistical analysis - Phylogenetic analysis

The most widely used method of estimating the reliability of trees is the nonparametric bootstrap. A drawback to the bootstrap method is that it can potentially be very time-consuming. For example, maximum likelihood is at present the most widely used statistical phylogenetic method, but because it is computationally intensive,

performing a bootstrap analysis on maximum likelihood trees can require prohibitive amounts of time.

In this thesis, analysis were performed using the Bayesian inference of phylogeny, which combines the prior probability of a phylogeny with the tree likelihood to produce a posterior probability distribution on trees [164]. The best estimate of the phylogeny can be selected as the tree with the highest posterior probability (i.e., the Maximum Posterior probability [MAP] tree) [307]. Topologies and branch lengths are not treated as parameters - as in ML methods [104] - but as random variables. Because posterior probabilities cannot be obtained analytically, they are approximated by numerical methods known as Markov chain Monte Carlo (MCMC) or Metropolis coupled MCMC (MCMCMC). These chains are designed to explore the posterior probability surface by integration over the space of model parameters. Trees are sampled at fixed intervals and the posterior probability of a given tree is approximated by the proportion of time that the chains visited it [419]. A consensus tree can be obtained from these sampled trees, and Bayesian posterior probabilities (PP) of individual clades, as expressed by the consensus indices, may be viewed as clade credibility values. Thus, Bayesian analysis of the initial matrix of taxa and characters produces both a MAP tree and estimates of uncertainty of its nodes, directly assessing substitution model, branch length, and topological variables, as well as clade reliability values, all in a reasonable computation time.

**Models of evolution for phylogenetic analysis**

Many different nucleotide substitution models have been developed for use in molecular evolutionary studies and mostly differ in the number of parameters involved. The simplest nucleotide substitution model has only one parameter, which is the probability of any one base changing to another [175]. A slightly more complex model has two parameters, one for the probability of a transversion occurring and a second for the probability of a transition occurring [186]. Kimura's two parameters model is one of the most commonly used models together with the Tajma-Nei (TN) or the Felsenstein (F81) model [104] and the Hasegawa, Kishino and Yano (HKY85) model [145], each

with 6 parameters and the general time-reversible (GTR) model with 10 parameters [201].

Most such analyses are performed at the DNA level. For protein-coding sequences, however, the DNA-based models are often too simplistic: for example, an A-G substitution in the second position of a tyr codon can have very different effects on the protein than the same substitution in the third position of a thr codon. Although some researchers have developed codon-based models [422], the most common model for protein evolution are substitution matrices derived from large, general sets of related proteins such as the Jones, Taylor and Thornton model (JTT) [174] and the WAG model [404]. It is recognised that different types of proteins are under different selective pressures and might not fit the most general models. Additional parameters can be considered, such as the gamma distribution parameter α, which defines the shape of the gamma distribution and accounts for the variation of substitution rates among sites. For the phylogenetic analysis of the *pol* and *env* nucleotide sequences of the SIV genome of *P.badius badius* we employed the General Time Reversible model of evolution with a gamma distribution of rates.

Retroviral and other reverse transcriptase (RT)-containing sequences may be subjected to unique evolutionary pressures, and models of molecular sequence evolution developed using other kinds of sequences may not be optimal. Distinctive features of RNA virus replication include high mutation rates, short generation times, large number of progeny, and frequent bottleneck events influencing local population sizes [94]. For the phylogenetic analysis on the amino- acid of the full-length genome of the *P.b. temminckii* we employed the rtREV model, which, compared to other matrices, yields higher likelihood values on a range of datasets including lentiviruses, spumaviruses, betaretroviruses, gammaretroviruses, and other elements containing reverse transcriptase. rtREV provides improvement over models which have been optimized on more general datasets, such as the JTT and WAG models and especially in cases where it is not feasible to adjust individual aminoacid substitution probabilities.

# 5. Microsatellite markers in seven monkey species from Taï National Park, Côte d'Ivoire: application to non-invasively collected samples

S. Locatelli[1,2,*], A.D. Roeder[2], M.W. Bruford[2]

*1-SwissTropical Institute (STI), University of Basel, Switzerland*
*2-Cardiff School of Biosciences, Cardiff University, CF1 3TL, UK*

*Keywords*: cross-species amplification, microsatellites, PCR, old world monkey, colobus

*Corresponding author mailing address: Swiss Tropical Institute
Socinstrasse 57
P.O. Box
CH-4002 Basel
Switzerland
Phone: +41 61 284 81 39
Fax: +41 61 284 81 05
Email: Sabrina.Locatelli@stud.unibas.ch

Running title: Microsatellite markers in seven monkey species

**This paper has been submitted to Folia Primatologica**

## 5.1 Abstract

In the frame of a non-invasive study aimed at estimating the prevalence of Simian immunodeficiency virus (SIV) infection by isolation of SIV viral RNA from faecal samples of habituated wild-living social groups of seven monkey species from Taï National Park, Côte d'Ivoire, we screened 16 human microsatellites to support and confirm visual identification of group members. Twenty-five to 37% of primers successfully amplified faecal extracted DNA samples from all species and produced reliable results. Although allelic variability and heterozygosity differed among species, the markers are informative and can be used in combination for individual discrimination.

## 5.2 Introduction

It is now well established that the human immunodeficiency viruses, HIV-1 and HIV-2, are the results of cross-species transmissions of simian immunodeficiency viruses (SIV) naturally infecting non-human primates in sub-Saharan Africa [63, 66, 122, 183, 338, 386] and that SIV transmission from primates to humans is most likely the result of contact with infected blood and tissues from primates hunted for bushmeat [143]. People continue to be exposed to a plethora of genetically diverse viruses through these practices [287] and it is therefore important not only to characterise new SIVs, but also to determine their prevalence in wild-living primate populations. It is possible to isolate SIVs in wild-living non-human primates non-invasively by extracting viral RNA from faecal samples [183, 219, 320, 386]. However, to ascribe a faecal sample to its owner is sometimes difficult in arboreal primate populations or in primate groups not habituated to the presence of human observers; therefore screening microsatellites could help to discriminate the faecal samples collected.

Faeces is now also a valuable source of non-invasively obtained DNA [363] and primate studies utilising faeces are prevalent although comprehensive wild population surveys using non-invasive samples remain rare [42]. Human microsatellites which amplify products in primates [72, 136] were tested in seven primate species from Taï forest. No specific primers exist for these species and the majority have never been

screened for cross-specific amplification. Although, some authors have underlined the difficulties of cross-amplification [58], a lack of high quality DNA for isolation of species specific markers meant screening for cross-specific amplification was the only option for obtaining microsatellite data for these species.

## 5.3 Materials and Methods

We collected faecal samples from 171 members of habituated groups of western red (*Piliocolobus badius badius*), black and white (*Colobus polykomos polykomos*) and olive colobus (*Procolobus verus*), Diana monkeys (*Cercopithecus diana*), Campbell's guenons (*Cercopithecus campbelli*), lesser (*Cercopithecus petaurista*) and greater spot-nosed monkeys (*Cercopithecus nictitans*) near the western border of the Taï Forest, in Côte d'Ivoire. These samples were collected in the same territory where sooty mangabeys have been found to harbour SIVsmm variants which are the ancestors of different groups of HIV-2, including those playing a major role in the HIV-2 epidemic in West Africa [322].

Freshly deposited faecal samples (2 to 5 g) were collected in 15 ml tubes filled with 7 ml of RNA*later* (Ambion, Austin, Texas) and stored at 4°C in the field and at -80°C in the laboratory. Genomic DNA was extracted using the QIAamp DNA stool minikit (Qiagen, Dusseldorf, Germany). Separate PCR amplifications at 16 microsatellite loci were carried out, in 10 µl using a Robocycler Gradient 96 (Stratagene, USA). The reaction contained 1 µl genomic DNA, 1x PCR buffer (Invitrogen, UK), 1–2 mM $MgCl_2$, 250 µM of each dNTP (ABgene, UK), 10 µM of each primer (MWG or TAGN, UK), 1µl Taq DNA polymerase (Invitrogen, UK), 0.4 mg/ml of BSA (Promega, USA) and 10% DMSO (Fisher Scientific, UK). D7s503, D11s925, D16s420, D17s791 were amplified as described in [72]. The PCR conditions were: 3 min at 95°C; 7 cycles of 45 s at 95°C, 1 min at 50°C; 90 s at 72°C; then 30 cycles with annealing temperature 54°C and finally 72°C for 10min. For D1s207, D2s141, D3s1768, D4s243, D5s1475, D6s271, D6s311, D6s501, DXs571, DXs8043, DXs6810, DXs6799 initial annealing was 48°C, followed by 40 cycles at 55°C.

Table 5.1: Sixteen human microsatellite loci screened using faecal DNA from seven primate species from Taï Forest, Côte d'Ivoire

| Locus | VNTR | PCR primer sequences 5'-3' | Fluorescent label | Marker References | Source of marker information |
|---|---|---|---|---|---|
| D1s207[a] | (CA)n | F:CACTTCTCCTTGAATCGCTT | - | Z16601 | Coote & Bruford (1996) |
| | | R:GCAAGTCCTGTTCCAAGTCT | - | | |
| D2s141[a] | (CA)n | F:ACTAATTACTACCCNCACTCCC | - | Z16793 | CHLC |
| | | R:TTTTCCAAACAGATACAGTGAACTT | - | | |
| D3s1768[c] | (GATA) | F:GGTTGCTGCCAAAGATTAGA | FAM | G08287 | CHLC |
| | | R:CACTGTGATTTGCTGTTGGA | - | | |
| D4s243[c] | (GATA) | F:AATCCCTTTTCTACCTTTCTATCAC | FAM | G33465 | Bayes et al. (2000) |
| | | R:GAGAGGAGAGATAAAAGATGTAAATG | - | | |
| D5s1475[c] | (GGAT)n | F:ACTCAAGCTAAGGCCTCAT | HEX | G08488 | Goossens et al. (2000) |
| | | R:GCATTTTGGGTCAAAAATTG | - | | |
| D6s271[b] | (CA)n | F:AAGGTAACAATTGGGAAATGGCTTA | NED | Z16648 | Coote & Bruford (1996) |
| | | R:TTACTTCATTATCTTAGCATACAGAG | - | | |
| D6s311[a] | (CA)n | F:TCATTGGTGTTGTGCATTAA | - | Z17200 | Coote & Bruford (1996) |
| | | R:TTGGAAGGATGAGAATTAAGG | - | | |
| D6s501[c] | (GATA)n | F:GCTGGAAACTGATAAGGGCT | NED | G08551 | Morin et al. (1998); Zhang et al (2001) |
| | | R:GCCACCCTGGCTAAGTTACT | - | | |
| D7s503[c] | (CA)n | F:ACTTGGAGTAATGGGAGCAG | HEX | Z16870 | Coote & Bruford (1996) |
| | | R:GTCCCTGAAAACCTTTAATCAG | - | | |
| D11s925[b] | (CA)n | F:AGAACCAAGGTCGTAAGTCCTG | HEX | Z17002 | Coote & Bruford (1996) |
| | | R:TTAGACCATTATGGGGGCAA | - | | |
| D16s420[b] | (CA)n | F:ATTTCCTGAGGTCTAAAGCA | FAM | Z17069 | Coote & Bruford (1996) |
| | | R:TTAGGCCCAGTCCACACTCA | - | | |
| D17s791[c] | (CA)n | F:GTTTTCTCCAGTTATTCCCC | NED | Z16689 | Coote & Bruford (1996) |
| | | R:GCTCGTCCTTTGGAAGAGTT | - | | |
| DXs571[a] | (CA)n | F:AATATTGGTGCAGGACTGT | - | Z17275.1 | CHLC |
| | | R:AATCAGATGCAGTGATGGGT | - | | |
| DXs8043[a] | (CA)n | F:AGTTCTCAGAAACATTTGGTTAGGC | - | Z53101 | CHLC |
| | | R:AATTATTGGCAAAGAGTACAGGCAG | - | | |
| DXs6810[a] | (GATA)n | F:ACAGAAAACCTTTTGGGACC | - | G09983 | CHLC |
| | | R:CCCAGCCCTGAATATTATCA | - | | |
| DXs6799[a] | (GATA)n | F:ATGAATTCAGAATTATCCTCATACC | - | G08099 | CHLC |
| | | R:GAACCAACCTGCTTTTCTGA | - | | |

a: Primers which failed to amplify from faecal samples (7/16); b: primers which amplified these loci, but which did not provide repeatable results (3/16); c: successful and reproducible amplifications (6/16). CHLC: Cooperative Human Linkage Centre

All sets of amplifications contained negative controls and gorilla and chimpanzee DNA as positive controls. At least one tissue-derived DNA sample per species was genotyped for the selected loci to verify the two sources had comparable allele sizes. For the initial screening, between two and six samples of each species were tested per locus and PCR products were electrophoresed on an agarose gel and visualised using

ethidium bromide. The 5' ends of the forward primers were fluorescently labelled as in Table 5.1 and PCRs using the Qiagen Multiplex kit were carried out following the manufacturers 'Microsatellite Protocol' for the reaction mix and thermocycling (annealing temperature: 57℃; number of cycles: 40). Three primer pairs (2 µM each primer) were multiplexed in a 10 µl reaction volume. A multi-tubes approach was followed to limit erroneous genotyping [115, 253, 363]. Products were separated using capillary electrophoresis (ABI 3100). Alleles were sized relative to an internal size standard (ROXHD400) using Gene Scan 3.7 (Perkin-Elmer). Alleles frequencies and expected heterozygosity were calculated using Genepop v 3.4 online [308].

## 5.4 Results

Of the 16 loci tested, three or more alleles were detected at six loci and these were used to genotype a total of 171 samples. Overall a total of 132 individuals were genotyped and the number of alleles per locus ranged from two to ten (Table 5.2a). Only in the case of black and white colobus could we compare genotypes between individuals of known relationship (i.e., mother-offspring) and here no deviation from Mendelian inheritance was observed. There were no instances in which more than two alleles were consistently observed per individual per locus, which suggests that amplification of contaminating mammalian, bacterial, or fungal DNA did not occur [37]. Genetic diversity is detailed in Table 5.2b. In several cases, observed exceeded the expected heterozygosity.

## 5.5 Discussion

One of the aims of this study was to screen a sufficient number of loci to enable us to discriminate samples collected for a Simian immunodeficiency virus infection study in social groups of different monkeys [219]. That we experienced some limitations using a set of microsatellite markers known to be efficient in other cercopithecines and colobines [72] emphasises the potential limitation of analyses conducted on some faecal samples and stresses the importance of extensively screening markers for population

genetic structure and mating system studies in wild-living, arboreal and semi-habituated monkey species.

## 5.6 Acknowledgements

We thank Dr. M. Peeters and Dr. M Bonhomme for the provision of high-quality DNA. S. Locatelli was supported by the Commision for Research Partnerships with Developing Countries, (KFPE), Bern, the Messerli foundation**,** Zürich**,** and the Guggenheim-Schnurr Foundation, Basel, Switzerland.

Table 5.2: Genotype outcome of six loci for 175 DNA samples (a) and microsatellite allele size, diversity and heterozygosity (b)

| **a** | | ***Piliocolobus badius badius (60)*** | ***Colobus polykomos polykomos (22)*** | ***Procolobus verus (11)*** | ***Cercopithecus diana (27)*** | ***Cercopithecus campbelli (7)*** | ***Cercopithecus petaurista (4)*** | ***Cercopithecus nictitans (3)*** |
|---|---|---|---|---|---|---|---|---|
| **N°samples analysed** | | 86 | 34 | 12 | 31 | 7 | 3 | 2 |
| **N°samples in replicates** | | 18 | 8 | 1 | 4 | 1 | 0 | 0 |
| **N°samples discarded (poor quality)** [a] | | 12 | 5 | 1 | 1 | 0 | 0 | 0 |
| **N°samples for statistical analysis** | | 56 F 4 T | 21 F 1 T | 10 F 1 T | 26 F 0 T | 6 F 1 T | 3 F 1 T | 2 F 1 T |
| **% of genotyping success** | | 86% | 85% | 92% | 97% | 100% | 100% | 100% |
| **b** | | | | | | | | |
| **D3s1768** | **AS** | 137-157 (4) | 145-161 (4) | 153-157 (2) | 191-219 (7) | 202-218 (4) | 195-209 (5) | 185-205 (4) |
| | **$H_O$** | 0.45 | 0.727 | 0.09 | 0.444 | 0.429 | 0.75 | 0.667 |
| | **$H_E$** | 0.381 | 0.512 | 0.09 | 0.7 | 0.584 | 0.786 | 0.8 |
| **D4s243** | **AS** | 141-201 (11) | 133-165 (9) | 145-177 (5) | no amp | 150-158 (2) | 204-208 (2) | 176-208 (4) |
| | **$H_O$** | 0.817 | 0.818 | 0.727 | - | 0.143 | 0.25 | 0.667 |
| | **$H_E$** | 0.833 | 0.718 | 0.533 | - | 0.143 | 0.25 | 0.667 |
| **D5s1475** | **AS** | 110-144 (11) | 98-146 (6) | 110-154 (8) | 100-128 (6) | 128-132(2) | 120-132 (4) | 120-132 (3) |
| | **$H_O$** | 0.85 | 0.954 | 1 | 0.926 | 0.857 | 0.5 | 1 |
| | **$H_E$** | 0.694 | 0.674 | 0.857 | 0.709 | 0.527 | 0.821 | 0.733 |
| **D6s501** | **AS** | * | * | 140-160 (6) | 140-176 (10) | 154-170 (6) | 143-167 (4) | 140-172 (5) |
| | **$H_O$** | - | - | 0.636 | 0.963 | 0.857 | 1 | 1 |
| | **$H_E$** | - | - | 0.545 | 0.829 | 0.813 | 0.821 | 0.933 |
| **D7s503** | **AS** | 134-154 (11) | 128-156 (7) | 132-140 (6) | 132-150 (10) | 128-134 (4) | 136-156 (6) | 136-152 (4) |
| | **$H_O$** | 0.9 | 0.636 | 1 | 0.852 | 1 | 0.75 | 0.667 |
| | **$H_E$** | 0.844 | 0.791 | 0.805 | 0.84 | 0.736 | 0.928 | 0.8 |
| **D17s791** | **AS** | 141-173 (11) | 157-167 (6) | 141-181 (7) | no amp | no amp | no amp | no amp |
| | **$H_O$** | 0.97 | 0.909 | 0.818 | - | - | - | - |
| | **$H_E$** | 0.886 | 0.776 | 0.713 | - | - | - | - |

a: less than three out six loci successfully amplified; F: faecal samples; T: tissue samples; $H_O$: Observed Heterozygosity; $H_E$: Expected Heterozygosity; AS: Allele sizes (bp)- (N°.alleles); *: Monomorphic; No amp: no amplificati on

# 6. Prevalence and genetic diversity of Simian immunodeficiency virus infection in wild-living red colobus monkeys (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire

Sabrina Locatelli[1], Florian Liegeois[1], Bénédicte Lafay[2], Amy D. Roeder[3], Michael W. Bruford[3], Pierre Formenty[4], Ronald Noë[5], Eric Delaporte[1], Martine Peeters[1*]

[1]UMR 145, Institut de Recherche pour le Développement (IRD), and University of Montpellier 1, Montpellier, France
[2]UMR CNRS-IRD 2724, Montpellier, France
[3]Cardiff School of Biosciences, Museum Avenue, Cardiff CF10 3TL, UK
[4]Ebola Taï Forest Project, Abidjan, Côte d'Ivoire; World Health Organisation (WHO), Department of Communicable Diseases, Surveillance and Response (CDS/CSR), Geneva, Switzerland
[5]Ethologie des Primates (DEPE-IPHC-CNRS UMR 7178) and University of Louis Pasteur, Strasbourg, France

*Corresponding author and address:
Martine Peeters
UMR 145, Institut de Recherche pour le Développement (IRD)
911 Avenue Agropolis, 34394 Montpellier, France.
Phone : (+33) 0467416297
Fax : (+33) 0467416146
E-mail : martine. peeters@mpl.ird.fr

**This paper has been published in**
***Infection, Genetics and Evolution* (2008), 8: 1-14**

## 6.1 Abstract

Numerous African primates are infected with Simian immunodeficiency viruses (SIVs). It is now well established that the clade of SIVs infecting West-central African chimpanzees (*Pan troglodytes troglodytes*) and western gorillas (*Gorilla gorilla gorilla*) represent the progenitors of Human Immunodeficiency Virus type 1 (HIV-1), whereas HIV-2 results from different cross-species transmissions of SIVsmm from sooty mangabeys (*Cercocebus atys atys*).

We present here the first molecular epidemiological survey of Simian Immunodeficiency Virus (SIVwrc) in wild-living western red colobus monkeys (*Piliocolobus badius badius*), which are frequently hunted by the human population and represent a favourite prey of western chimpanzees (*Pan troglodytes verus*). We collected faecal samples (n=88) and we assessed individual discrimination by microsatellite analyses and visual observation. We tested the inferred 53 adult individuals belonging to two neighbouring habituated groups for presence of SIVwrc infection by viral RNA (vRNA) detection. We amplified viral polymerase (*pol*) (650 bp) and/or envelope (*env*) (570 bp) sequences in 14 individuals, resulting in a minimal prevalence of 26% among the individuals sampled, possibly reaching 50% when considering the relatively low sensitivity of viral RNA detection in faecal samples. With a few exceptions, phylogenetic analysis of *pol* and *env* sequences revealed a low degree of intragroup genetic diversity and a general viral clustering related to the social group of origin. However, we found a higher intergroup diversity. Behavioural and demographic data collected previously from these communities indicate that red colobus monkeys live in promiscuous multi-male societies, where females leave their natal group at the subadult stage of their lives and where extra-group copulations or male immigration have been rarely observed. The phylogenetic data we obtained seem to reflect these behavioural characteristics. Overall, our results indicate that wild-living red colobus represent a substantial reservoir of SIVwrc. Moreover, because of their frequent association with other monkey species, the predation pressure exerted by chimpanzees (*Pan troglodytes verus*) and by poachers around and inside the park, simian to simian and simian to human SIVwrc cross-species transmission cannot be excluded.

**Keywords:** SIV; HIV; red colobus; faecal samples; microsatellites; non-invasive sample collection; Côte d'Ivoire; polyspecific associations; bushmeat; Taï Forest.

## 6.2 Introduction

Serological evidence for Simian Immunodeficiency Virus (SIV) infection has been identified, to date, in 39 different nonhuman primate (NHP) species in sub-Saharan Africa. SIV infection has been molecularly confirmed in 32 NHP species and in 19, full length SIV sequences were obtained [382]. High genetic diversity is observed among the known SIVs, but, generally, each primate species is infected with a species-specific virus that forms monophyletic lineages in phylogenetic trees [27, 78]. In addition, several "major SIV lineages" have been identified, which represent groups of SIVs from different primate species that are more closely related to one another than they are to other SIVs. For some of these SIV lineages, virus and host phylogenies seem to match, suggesting virus/host co-speciation, but there are also numerous examples of cross-species transmissions and recombination [14, 21, 28, 29, 151, 162, 168, 169, 318, 390]. Interestingly, it has also been shown that one primate species can be infected with two different SIVs [1, 350]. One of the most striking examples of cross-species transmission, followed by recombination, is SIVcpz in chimpanzees from central Africa (*Pan troglodytes troglodytes*), with its 5' end being closest to that of SIVrcm from red capped mangabeys and the 3' end most closely related to those of the SIVgsn/SIVmus/SIVmon lineage from greater spot nosed, mustached and mona monkeys [14, 23, 79, 323]. Apparently, chimpanzees acquired their SIV infection through hunting other NHP species [242, 338]. With the exception of SIVcpz from chimpanzees and SIVgor from gorillas, all SIVs identified so far originate from primates belonging to the family *Cercopithecidae*, or Old World monkeys, which is subdivided into two subfamilies: *Colobinae* and *Cercopithecinae* [93]. The colobinae subfamily comprises three genera, *Colobus*, *Procolobus* and *Piliocolobus* [139]. The first primate lentivirus identified in the *Colobinae* subfamily, SIVcol from mantled guerezas (*Colobus guereza*) in Cameroon, forms a separate divergent lineage in the phylogenetic tree analysis [78]. Subsequently, SIVwrc and SIVolc were identified in western red (*Piliocolobus badius badius*) and in olive colobus (*Procolobus verus*) respectively, in Taï National Park, Côte d'Ivoire.

Phylogenetic analyses of a 2000 bp fragment in the *pol* region showed that SIVwrc and SIVolc sequences each formed species specific monophyletic lineages, but were not at all related to the SIVcol strain obtained from a mantled guereza in Cameroon [77].

We know today that handling of infected NHP carcasses exposes the human population to a risk of transmission of different viruses, including SIV [287]. Today, apart from SIVsmm in sooty mangabeys (*Cercocebus atys atys*), which is recognised as the progenitor of Human Immunodeficiency virus type 2 (HIV-2) [63, 66], we know of SIVcpz and SIVgor from chimpanzees (*Pan troglodytes troglodytes*) from western gorillas (*Gorilla gorilla gorilla* ) inhabiting West-central Africa to have given rise to human immunodeficiency virus type 1 (HIV-1), group M, N and O [122, 183, 338, 386]. Moreover, it has been shown that SIVs isolated in sooty mangabeys from the Taï Forest are closely related to certain HIV-2 variants playing a role in the west African HIV-2 epidemic [322]. The Taï Forest is home to nine diurnal primate species, including chimpanzees, western red colobus, olive colobus, black and white colobus, different species of guenons (Diana monkeys, Campbell's monkeys, lesser spot-nosed and greater spot-nosed monkeys) and sooty mangabeys [240]. The red colobus (*Piliocolobus badius badius*) and the Diana monkey (*Cercopithecus diana diana*) are the primate species best represented in Taï National Park, but, after the antelopes, they also are the most extensively hunted by the human population [310]. In addition, chimpanzees (*Pan troglodytes verus*) of the Taï Forest are also known to hunt western red colobus monkeys frequently [33]. More than 1400 chimpanzees issued from U.S. research centers and zoos (18.16% were African born) have been tested for HIV cross-reactive antibodies, yet no SIV infection has been identified in the chimpanzee subspecies *P.t.verus* [362]. However, in analogy to the origin of SIVcpz in chimpanzees from central Africa, it cannot be excluded that western chimpanzees became infected with a SIV harboured by their prey. It is important to note that these animals have been screened with HIV assays, which may not be sensitive enough to detect infections with divergent SIVs. Moreover, another retrovirus, the simian T-cell leukaemia virus type 1 (STLV-1), is already circulating in both western red colobus and chimpanzees in the Taï Forest [209].

In order to investigate the likelihood of West African chimpanzees becoming infected with SIVwrc from western red colobus monkeys and to estimate the risk of SIVwrc transmission into the human population, it is important to gain more information on SIV infection in this primate species in its natural habitat. The Taï National Park provided a unique opportunity to investigate SIVwrc infection in wild-living western red colobus (*Piliocolobus badius badius)*. Their ecological and behavioural characteristics have been studied extensively since 1992 by researchers who habituated several social groups to the presence of human observers [240]. In this study, we collected faecal samples from two habituated free-ranging red colobus groups to study the prevalence and genetic diversity of SIVwrc in his natural hosts.

## 6.3 Materials and Methods

**Animals and sample collection**

The present study was carried out on two groups of habituated red colobus monkeys (Figure 6.1), named Bad1b and Bad2a, inhabiting the Taï National Park. The park measures 4500 $km^2$ and it is located in the south-western part of Côte d'Ivoire (6°20'N to 5°10'N and 4°20W to 6°50'W). The Taï For est represents the largest block of protected West African rainforest and is considered a biodiversity hotspot within the upper Guinean forest region. The study site is located near the western border of the park, at about 20 km south-east of the town of Taï and it has been previously described [358, 410]. We identified individuals based on facial features, pelage colour, permanent marking from fights and shape and length of the tail. Due to their relative similar body size, adult males were discriminated from females by observing the presence or absence of sexual swellings, copulation calls, presenting behaviours or large testes [269]. Sampling was biased toward adults versus juveniles or infants because of more distinctive physical features and toward males versus females because males were less wary of our presence and therefore more easily identified. We collected data on group size, composition and range in parallel to the faecal sampling performed from March 2004 through July 2004. We estimated the mean group size of Bad1b and Bad2a to be around 60 individuals per group, including juveniles and infants. Groups' composition and size were consistent with previous studies [119, 191].

In 2004, each group occupied a territory ranging from 50 to 70 ha, with slight overlap toward the east of the grid. To avoid any misinterpretation, samples were not collected or considered, whenever the two groups were in proximity. During group observation, we collected freshly deposited faecal samples (2 to 5 g) into 15 ml tubes filled with 7 ml of RNA*later* (Ambion, Austin, Texas, USA). We recorded the name of the collector, the name, sex and age class of the individual (if known) as well as the date, time and location on a painted 3 $km^2$ grid system with 100 x 100 m cells for each faecal sample. The grid system in the field consisted of lines in north-south direction (trees marked with blue paint) and lines in east-west direction (trees marked with yellow paint). Every north to south line was characterised by a letter and every east-west line by a number. This way, every cell can be identified by a precise code (for example L 8) (Figure 6.1). Samples were stored at camp for 30 to 60 days at 4°C and subsequently shipped to the laboratory in Montpellier, France. Upon receipt samples were stored at -80°C.

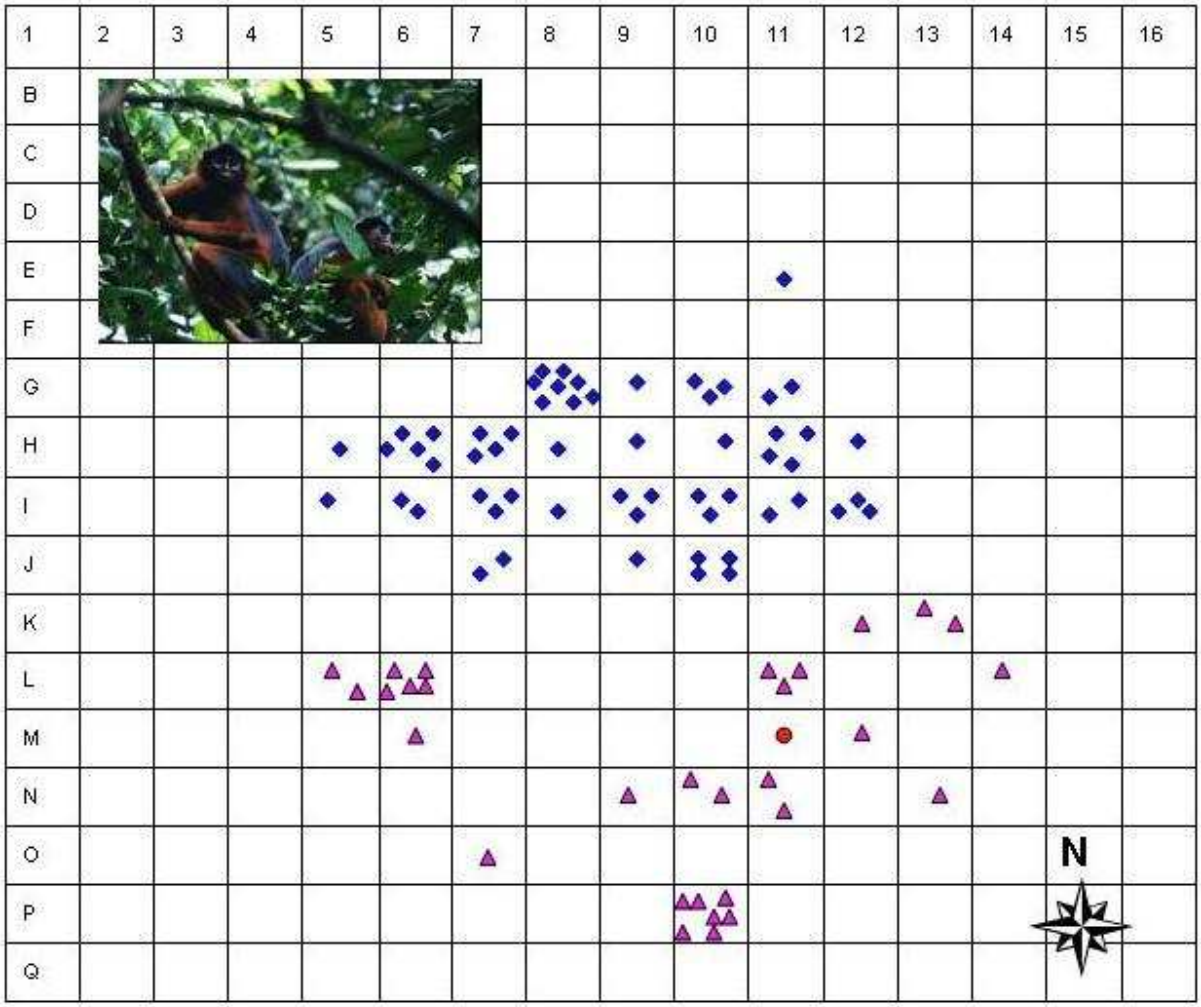



Figure 6.1: western red colobus (*Piliocolobus badius badius*) from the Taï Forest (Photo taken by F. Möllers). Localisation of faecal samples collected for the two social groups Bad1b (pink rhomboids) and Bad2a (blue triangles) and for the previously collected blood sample (red dot) on a painted 3 km2 grid system with 100 x 100 m cells.

**Detection of HIV cross-reactive antibodies in red colobus faecal samples**

We recovered IgGs after dialyses of faecal samples by applying the methods previously described for antibody detection in faecal samples of gorillas and chimpanzees [183, 386]. About 1.5 ml of faecal sample was filtered, potential viruses were inactivated and then the samples were dialysed to eliminate the presence of salt contained in the RNA*later* medium. We then tested the resulting sample for HIV cross-reactive antibodies by the INNO-LIA HIV confirmation test (Innogenetics, Ghent,

Belgium). This assay has proved to be useful in identifying new SIVs, notably SIVwrc and SIVolc, in infected blood samples [77]. This test includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip. The five HIV-1 antigens are synthetic peptides for the exterior envelope glycoprotein (sgp 120), as well as recombinant proteins for the transmembrane envelope glycoprotein (gp41), integrase (p31), core (p24), and matrix (p17) proteins. HIV-1 group O envelope peptides are included in the HIV-1 sgp120 band. The HIV-2 antigens include synthetic peptides for sgp105, as well as recombinant gp36 protein. In addition to these HIV antigens, each strip has control lines: one sample addition line (3+) containing anti-human immunoglobulin (IgG) and two test performance lines (1+ and +/-) containing human IgG. We performed all assays according to manufacturer's instructions, with alkaline phosphatase-labelled goat anti-human IgG as the secondary antibody. Samples should be scored as INNO-LIA positive when they react with at least one HIV antigen and have a band intensity equal to or greater than the assay cutoff (+/-) lane; samples that react less strongly, but still visibly with two or more HIV antigens should be classified as indeterminate; and samples reacting with no bands or only one band with less than +/- intensity should be classified as negative.

**Nucleic acid extraction from western red colobus faecal samples**

We extracted viral RNA from faecal samples using the RNAqueous-Midi kit (Ambion, Austin, Texas, USA) as previously described [183, 321]. Briefly, 6 ml of lysis-binding solution was added to 1.5 ml of faecal sample solution and vortexed vigorously until the sample was thoroughly homogenised. The suspension was clarified by centrifugation (at 4500 RPM for 5 min) and an equal volume of 64% ethanol was added. The solution was passed through a glass fibre filter unit to bind nucleic acids and washed three times with washing buffer. The nucleic acids were then eluted (1200 µl) and subsequently precipitated with LiCl and spun at 13000 RPM. The resulting pellet was washed once with cold 70% ethanol, air dried, resuspended in 50 µl of RNase free-water and then stored at -80℃.

We extracted faecal DNA using the QIAamp Stool DNA mini kit (QIAGEN, Hilden, Germany). Briefly, 2 ml of faecal RNA*later* mixture were centrifuged and the pellet was

resuspended in stool lysis buffer, clarified and passed through a DNA binding column. Bound DNA was eluted in 100 µl storage solution and stored at -20℃.

**Microsatellite analyses**

Analyses of microsatellite loci were performed in order to determine which faecal samples were duplicates from the same individual. We used primers for microsatellite loci which have originally been isolated from human DNA and that were found to yield amplification products in several primate species [72, 135]. The amplification reactions (total volume = 10 µl) were composed of 1 µl genomic DNA (amount of DNA varying from 6ng up to 85ng/ µl), 1X PCR buffer (Invitrogen, UK), 1 to 2 mM $MgCl_2$, 250 µM of each dNTP (ABgene, UK), 10 µM of each primer (MWG or TAGN, UK), 1 µl Taq DNA polymerase (Invitrogen, UK), 0.4 mg/ml of BSA (Promega, USA), 10% DMSO (Fisher Scientific, UK). PCR amplifications were carried out on a Robocycler Gradient 96 (Stratagene, USA). The loci D7s503 and D17s791 were amplified as previously described [72]. Briefly, a 3 min denaturation at 95° followed by seven cycles of 45 s at 95℃, 1 min at 50℃, and 90 s at 72℃. This was fol lowed by a further 30 cycles with the annealing temperature adjusted to 54℃ and a final 10 min extension step at 72℃. For the remaining loci (D4s243, D5s1475), we applied the same conditions except for the annealing temperatures raising from 48℃ to 55℃ th roughout 40 cycles. All sets of amplifications contained gorilla and chimpanzee DNA positive controls to confirm success of the PCR and multiple negative controls to monitor contamination. The 5'-end of the selected forward primers were fluorescently labelled (FAM-D4s243, HEX-D5s1475, HEX-D7s503, NED-D17s791) (MWG and Applied Biosystems, UK) and PCRs using the Multiplex Qiagen kit (Qiagen, Hilden, Germany) were carried out following the 'Microsatellite Protocol' (available in the Qiagen multiplex PCR handbook) for the reaction mix and the cycling program. Primers were used at 2 µM each. Amplification products were separated using capillary electrophoresis on a ABI 3100 genetic analyzer (Applied Biosystems, UK). Alleles were sized relative to an internal size standard (ROXHD400) using Gene Scan 3.7 (Perkin-Elmer Applied Biosystems, UK). We followed a multi-tube approach whenever possible, with at least seven independent positive PCR reactions confirming a homozygote and at least three reactions confirming

both bands of a heterozygote [115, 253, 363]. All faecal samples that yielded SIV positive results were retested to reconfirm their genotype. This procedure was applied to prevent incorrect genotyping due to stochastic amplification of only one of two possible alleles (allelic dropout). We extracted DNA from hair from two researchers involved in this study and genotyped at each locus to detect possible human contamination at both the sample collection and PCR stage [4, 395]. We also relied on a few high-quality DNA samples as a species control mostly to verify whether the two sources had comparable allele sizes.

**Amplification of SIVwrc sequences from faecal RNA**

We performed RT-PCR amplification of faecal virion using two sets of primers specific for SIVwrc *pol* and *env* sequences. We designed the *pol* primers on the basis of the sequence alignment of 5 previously published sequences, 4 SIVwrc from western red colobus and one SIVolc from an olive colobus [77]. In turn, the *env* primers were designed only on the basis of two SIVwrc sequences that have been fully molecularly characterised (Liegeois et al., manuscript in preparation). The regions amplified correspond to the 3' end of the *pol* gene and the gp41 region of the *env* gene. cDNA was synthetised using the wrcpolR1/wrcenvR1 primers followed by nested PCRs using primers F1/R1 and F2/R2 as inner and outer primers respectively. The *pol* primers included wrcpolF1 (5'-TAGGGACAGAAAGT ATAGTAATHTGG-3') and wrcpolR1 (5'-GCCATWGCYAATGCTGTTTC-3') as outer primers and wrcpolF2 (5'AGAGAC AGTA AGGAAGGGAAAGCAGG-3') and wrcpolR2 (5'-GTTCWATTCCTAACCAC CAGCADA-3') as inner primers for the second PCR round. The *env* primers included wrcenvF1 (5'-TGGCAGTGGGACAAAAATATAAAC-3'), wrcenvR1 (5'-CTGGCAGTCCCTCTTCCAAG TTGT-3'), wrcenvF2 (5'-TGATAGGGMTGGCTCCTGGTGATG-3') and wrcenvR2 (5'-AATCCCCATTTYAACCAGTTCCA-3').

We performed PCRs using a Long Expand PCR kit (Roche Molecular Biochemicals, Manheim, Germany) under the following conditions: a hot start at 94℃ for 2 min followed by 10 cycles of denaturation at 92℃ for 20 s, annealing at 45℃ for 45 s, extension at 72℃ for 1.5 min, and 20 cycles with the annealing temperature increased to 50℃ with extension at 72℃ for 1.5 min. Amplification was completed by a final

extension at 72℃ for 5 min. PCR conditions for the second PCR round were the same except that the extension time during cycling was 45 s. RT-PCR products from *pol* (~650 bp) and *env* (~570 bp) regions were purified (Q-Biogene, Illkirch, France), and directly sequenced using the inner (F2/R2) primers on an ABI 3130xl Genetic Analyser (Applied Biosystem, Courtaboeuf, France). We then checked and assembled the sequences using the software package Lasergene (DNASTAR Inc. Madison, USA).

**Phylogenetic analysis**

We aligned the SIVwrc sequences in *pol* and *env* with previously published SIVwrc sequences and a set of reference sequences from different SIV lineages. Sites that could not be unambiguously aligned were excluded from the analyses. Neither *pol* nor *env* sequences showed substitution saturation as evidenced by the comparison of transitional versus transversional distances between all possible sequence pairs. The model of evolution for *pol* and *env* (general time reversible model of evolution with a gamma distribution of rates) was selected under the Akaike information criterion using Modeltest v3.7 [302]. We performed Bayesian inferences using MrBayes v3.1.2 [313]. The tree-space was explored using four chains over 1,000,000 generations sampled every 100. Burn-in value was fixed at 10% of the total generation number after empirical determination of the convergence. We examined Bayesian parameters with the Tracer program (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) and all estimated sample sizes were greater than 1621. *Pol* and *env* phylogenies of red colobus monkeys' SIV were rooted using sequences of corresponding regions in SIVlho from l'Hoest monkeys (*Cercopithecus lhoesti*) (AF188114), SIVsun from sun-tailed monkeys (*Cercopithecus solatus*) (AF131870) and SIVmnd-1 from mandrills (*Mandrillus sphinx*) (M27470) as well as SIVolc from olive colobus (*Procolobus verus*) (accession and manuscript in preparation).

### Nucleotide sequence accession numbers

EMBL accession numbers (in parentheses) for the sequences determined in this study are as follows for the *pol* gene: SIVwrc-04CI-196 (AM743109), SIVwrc-04CI-116 (AM743110), SIVwrc-04CI-299 (AM743111), SIVwrc-04CI-110 (AM743112), SIVwrc-04CI-115 (AM743113), SIVwrc-04CI-112 (AM743114), SIVwrc-04CI-223 (AM743115), SIVwrc-04CI-52 (AM743116), SIVwrc-04CI-237 (AM743117); and for the *env* gene: SIVwrc-04CI-268 (AM743118), SIVwrc-04CI-52 (AM743119), SIVwrc-04CI-116 (AM743120), SIVwrc-04CI-110 (AM743121), SIVwrc-04CI-237 (AM743122), SIVwrc-04CI-175 (AM743123), SIVwrc-04CI-296 (AM743124), SIVwrc-04CI-223 (AM743125), SIVwrc-04CI-299 (AM743126), SIVwrc-04CI-280 (AM743127), SIVwrc-04CI-32 (AM743128), SIVwrc-04CI-196 (AM743129), SIVwrc-04CI-112 (AM743130).

## 6.4 Results

### Samples and animal identification

We collected a total of 88 faecal samples between March and July 2004, 30 from the Bad1b social group and 58 from Bad2a. Although several individuals were visually recognized in both groups, we used microsatellite analysis for definitive individual identification. Unlike several other primate species in which multiple microsatellite loci are well characterised, there are no published microsatellite data for red colobus monkeys. Due to the lack of DNA of suitable quality to construct microsatellites libraries, cross-specific amplification was determined to be the most suitable method to find polymorphic microsatellites loci in these species. Nine human microsatellite loci that were found to yield amplification products in other primate species were screened for amplification in red colobus (Locatelli et al. submitted). We subsequently chose four highly polymorphic markers (D4s243, D5s1475, D7s503 and D17s791). Amplicon sizes for each of the loci are as follows: D4s243 (141-201 bp), D5s1475 (110-142 bp) D7s503 (130-154 bp) D17s791 (141-173 bp). The loci provided enough information to exclude sample misidentification. The microsatellite analysis results are summarised in table 5.1. Twelve out of 88 samples yielded poor or no microsatellite data and three samples were not genotyped due to insufficient material. Among the remaining 73 samples, 54 were

confirmed to originate from different individuals. However, for one individual (sample 211) we were not able to perform RNA extraction due to insufficient material available, therefore we considered the number of individuals tested to be 53. Twelve samples from 12 different individuals were confirmed to have been collected in duplicate and 3 samples to have been collected three times from the same individual at different times of the day or on different days. We collected samples from 24 individuals from the Bad1b group, which by visual inspection corresponded to 11 males, 8 females, and 5 individuals with undetermined gender. Similarly, in the Bad2a group we collected samples from 21 males, 4 females and 5 individuals with undetermined gender (30 individuals in total). Given the average size of each group to be around 60 individuals, we estimated that between 68% and 83% of the adult population had been sampled.

**Detection of SIVwrc antibodies**

The first SIVwrc positive animals were identified by using the Innolia HIV confirmation assay [77, 294]. Blood samples from red colobus showed clear IgG bands and cross-reacted with HIV antigens for the core protein p24 or the matrix protein p17. Consequently, and in analogy with studies on wild living chimpanzees and gorillas, we also tested 67 faecal samples (24 samples corresponding to 22 individuals from group Bad1b and 43 samples corresponding to 28 individuals from group Bad2a) from 50 out of 54 individuals with the same assay; the remaining 4 individuals were not tested because of insufficient material availability. Only one sample revealed a clear presence of IgG, but did not react with any of the HIV antigens. The samples from the remaining 49 individuals had to be considered ‘not interpretable’, since the baseline results did not fit the requirements, i.e., the anti human IgG upper line was absent (n=22) or gave only a weaker (n=27) signal than that of the lower human IgG line on the strips, and no reactivity with any HIV antigens was observed (Table 6.1).

Table 6.1: Genotype results at four loci and SIV results for all samples collected

| [a]Faecal spl.N° | Social group | [b]Sex | [c]Locus D4s243 | Locus D5s1457 | Locus D7s503 | Locus D17s791 | [d]Assigned indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *pol* | *env* |
| 26 | Bad1b | nd | ns | ns | ns | ns | bs | nd | nd | |
| 27 | Bad1b | nd | 157157 | ns | 142146 | 161165 | 1 | nd | - | - |
| 28 | Bad1b | nd | 145169 | 122126 | 146154 | 153163 | 2 | NI | - | - |
| 29 | Bad1b | nd | ns | 118126 | 138146 | 155161 | 3 | NI | - | - |
| 30 | Bad1b | nd | 145157 | ns | ns | 141157 | 4 | nd | - | - |
| 31 | Bad1b | nd | 145157 | 122126 | 148148 | 141157 | 4 | NI | - | - |
| 32 | Bad1b | nd | 161161 | 126130 | 134140 | 157163 | 5 | NI | - | + |
| 52 | Bad1b | M | 145161 | 126134 | 142144 | 147161 | 13 | NI | + | + |
| 94 | Bad2a | M | 161173 | 126130 | 140140 | 153171 | 47 | nd | - | - |
| 95 | Bad2a | M | 161201 | 122126 | 140150 | 153161 | 25 | nd | - | - |
| 96 | Bad2a | M | ns | 122130 | ns | 151165 | bs | nd | nd | |
| 97 | Bad2a | M | 173201 | ns | 140152 | 153171 | 26 | nd | - | - |
| 102 | Bad2a | M | ns | ns | ns | ns | bs | NI | nd | |
| 103 | Bad2a | M | ns | ns | 146150 | ns | 27 | NI | - | - |
| 107 | Bad2a | nd | 153165 | 122130 | 144150 | 151159 | 41 | NI | - | - |
| 108 | Bad2a | F | nd | nd | nd | nd | nd | nd | nd | |
| 108b | Bad2a | nd | ns | ns | ns | ns | bs | NI | nd | |
| 109 | Bad2a | M | 161165 | 122126 | 146150 | 151153 | 27 | nd | - | - |
| 110 | Bad2a | nd | 153161 | 122126 | 150152 | 153165 | 42 | NI | + | + |
| 111 | Bad2a | M | 161201 | 122126 | 140150 | 153161 | 25 | NI | - | - |
| 112 | Bad2a | nd | 145173 | ns | 142150 | 149167 | 43 | NI | + | + |
| 115 | Bad2a | M | 153161 | 122126 | 140150 | 153165 | 49 | NI | + | - |
| 116 | Bad2a | M | 145165 | 126134 | 140150 | 153161 | 28 | nd | + | + |
| 118 | Bad2a | M | 189197 | ns | 138152 | ns | 33 | NI | - | - |
| 119 | Bad2a | M | 161165 | 122126 | 146150 | 151153 | 27 | NI | - | - |
| 120 | Bad2a | M | 201201 | 126130 | 140146 | 161171 | 29 | nd | - | - |
| 126 | Bad2a | M | ns | 122130 | ns | ns | bs | NI | nd | |
| 127 | Bad2a | M | 173185 | 126130 | 140148 | 159171 | 50 | nd | - | - |
| 128 | Bad2a | nd | 145161 | 114118 | 140154 | 165171 | 44 | NI | - | - |
| 129 | Bad2a | M | 161201 | 126134 | ns | 153161 | 25 | NI | - | - |
| 130 | Bad2a | nd | 141161 | ns | 140150 | 153161 | 45 | NI | - | - |
| 167 | Bad2a | M | 145165 | 126134 | 140150 | 153161 | 28 | NEG | - | - |
| 169 | Bad1b | M | 145149 | 122134 | 142146 | 149163 | 17 | NI | - | - |
| 171 | Bad2a | M | 153161 | 122126 | 140150 | 153165 | 49 | NI | - | - |
| 172 | Bad2a | M | 161173 | 126130 | 130140 | 153171 | 30 | NI | - | - |
| 174 | Bad2a | M | 189189 | 122130 | 138140 | 151165 | 35 | nd | nd | |
| 175 | Bad2a | F | 153165 | 122130 | 144150 | 151159 | 41 | NI | - | + |
| 176 | Bad2a | M | 173201 | 126130 | 140152 | 153171 | 26 | NI | nd | |
| 177 | Bad2a | M | 161173 | 126130 | 140148 | 153171 | 47 | NI | - | - |
| 178 | Bad2a | M | 149173 | 126134 | 140150 | 153171 | 31 | NI | - | - |
| 179 | Bad2a | M | ns | 126130 | ns | ns | bs | NI | nd | |
| 182 | Bad2a | M | 161173 | 126126 | 140140 | 153171 | 34 | NI | - | - |
| 190 | Bad2a | M | 153161 | 122126 | 140150 | 153165 | 49 | nd | - | - |
| 195 | Bad1b | F | 157169 | 122130 | 140146 | 167169 | 11 | NI | - | - |
| 196 | Bad1b | nd | 157169 | nt | 144148 | 151159 | 6 | NI | + | + |
| 198 | Bad1b | F | 157157 | 119127 | ns | 157161 | 12 | NI | - | - |
| 206 | Bad2a | M | 189197 | 122130 | 140148 | 151165 | 37 | NI | - | - |
| 211 | Bad2a | nd | 149161 | 126144 | 138140 | 141153 | 54 | NI | nd | |

Table 6.1: Genotype results at four loci and SIV results for all samples collected – continued

| [a]Faecal spl.N° | Social group | [b]Sex | [c]Locus D4s243 | Locus D5s1457 | Locus D7s503 | Locus D17s791 | [d]Assigned indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *pol* | *env* |
| 213 | Bad2a | M | 201201 | 126130 | 140146 | 161171 | 29 | NI | - | - |
| 215 | Bad2a | M | nd | nd | nd | nd | nd | NI | nd | |
| 223 | Bad1b | M | 145157 | 114122 | 146154 | 167169 | 9 | NI | + | + |
| 229 | Bad2a | M | 145161 | 122122 | 138152 | 157165 | 32 | NI | - | - |
| 230 | Bad2a | M | nd | nd | nd | nd | nd | nd | nd | |
| 231 | Bad2a | M | 145153 | ns | 140154 | ns | 38 | NI | - | - |
| 236 | Bad2a | M | 161173 | 118122 | 140150 | 153163 | 36 | nd | - | - |
| 237 | Bad2a | M | 157165 | 126130 | 140148 | 161167 | 48 | NI | + | + |
| 241 | Bad2a | F | 145153 | 118122 | 142154 | 161173 | 51 | NI | - | - |
| 243 | Bad1b | F | ns | 126130 | 140150 | 147159 | 20 | NI | - | - |
| 247 | Bad1b | F | 157169 | 126130 | 140146 | 151159 | 18 | NI | - | - |
| 248 | Bad2a | M | 189189 | 122130 | 138140 | 151165 | 35 | nd | - | - |
| 249 | Bad2a | M | 189197 | ns | 138152 | 155165 | 33 | NI | - | - |
| 250 | Bad2a | M | 161173 | 118122 | 140150 | 153163 | 36 | NI | - | - |
| 251 | Bad2a | M | ns | ns | ns | ns | bs | NI | nd | |
| 256 | Bad2a | M | 161173 | 118122 | 140150 | 153163 | 36 | NI | - | - |
| 257 | Bad2a | M | ns | ns | ns | ns | bs | NI | nd | |
| 258 | Bad2a | M | 161173 | 126126 | 140140 | 153171 | 34 | NI | - | - |
| 260 | Bad2a | M | ns | 122126 | ns | ns | bs | NI | nd | |
| 261 | Bad2a | M | 189189 | 122130 | 138140 | 151165 | 35 | NI | - | - |
| 262 | Bad2a | M | 157161 | 122126 | 140150 | 153165 | 39 | NI | - | - |
| 263 | Bad2a | M | ns | 126130 | ns | ns | bs | nd | nd | |
| 264 | Bad2a | M | 173201 | 122126 | 144150 | 153161 | 40 | NI | - | - |
| 267 | Bad1b | F | ns | 118126 | 138146 | 155161 | 19 | nd | - | - |
| 268 | Bad1b | F | 145145 | 122126 | 138150 | 163167 | 16 | NI | - | + |
| 269 | Bad1b | M | ns | ns | ns | ns | bs | NI | nd | |
| 270 | Bad1b | M | 157169 | 122130 | 140146 | 167169 | 11 | nd | - | - |
| 274 | Bad1b | F | 157157 | 122126 | 142146 | 161165 | 1 | NI | - | - |
| 275 | Bad1b | F | ns | ns | ns | ns | bs | NI | nd | |
| 276 | Bad1b | M | 145169 | 126130 | 140150 | 149161 | 10 | NI | - | - |
| 280 | Bad1b | M | 157169 | 126130 | 144146 | 151159 | 22 | NI | - | + |
| 281 | Bad1b | M | 175191 | 122130 | 144144 | 147165 | 7 | NI | - | - |
| 282 | Bad1b | M | 157169 | 126130 | 144144 | 151 | 8 | nd | - | - |
| 290 | Bad2a | F | 145157 | 110118 | 142152 | 163165 | 52 | NI | - | - |
| 292 | Bad2a | F | 145185 | ns | 140148 | 147159 | 53 | NI | - | - |
| 293 | Bad1b | M | 169181 | 126130 | 150154 | 159173 | 23 | NI | - | - |
| 296 | Bad1b | M | 173189 | 122126 | 150152 | 147167 | 24 | NI | - | + |
| 299 | Bad1b | M | 145157 | 122126 | 142148 | 145167 | 14 | NI | + | + |
| 300 | Bad1b | F | ns | 122126 | 138146 | 161165 | 21 | NI | - | - |
| 301 | Bad1b | M | 145157 | 122130 | 140146 | 167169 | 15 | NI | - | - |

[a] Number given to the sample collected.
[b] M, male; F, female; nd, not determined
[c] Genotype analysis: ns, not scorable; nd, not determined
[d] Individual number, number assigned to every sample which shows the same genetic profile (conservative method) bs, 'bad sample', a sample which DNA quality was not sufficient to provide repeatable and reliable genotype results
[e] NI, not interpretable: absence or too low IgG bands; nd, not determined; NEG, negative
[f] *pol* and *env* PCR amplification results: +, amplification; -, no amplification ; nd, not determined

**Viral RNA detection of SIVwrc**

We extracted RNA from 73 faecal samples belonging to 53 distinct individuals: 24 were members of the Bad1b group and 29 were members of the Bad2a group. RNA was then subjected to RT-PCR analysis using two sets of consensus primers, which amplified a ~650-bp fragment in *pol* and a ~570-bp fragment in the *env* regions. This analysis identified 14 SIVwrc infected individuals, 8 in group Bad1b and 6 in group Bad2a using one or both primer sets (Table 6.1 and Table 6.2). Sequence analysis of the respective amplification products confirmed infection, and showed that the animals were infected with genetically different SIVwrc strains. We were able to amplify the *env* fragment in 13 samples and the *pol* fragment in 9 out of 14 samples, which corresponds in this sample set to a PCR efficacy of 93% and 64% respectively. Altogether, 26% of the individuals tested were SIVwrc positive, 30% in Bad1b and 21% in Bad2a. RNA extraction was performed in duplicate, when there was enough material available (n=19) and for one sample, RNA extraction was repeated three times. In 16 samples, PCRs from repeated RNA extractions gave consistent results for both *pol* and *env* fragments (14 SIVwrc negative and 2 positive). The remaining 4 samples, which proved to be SIVwrc positive in the first RNA extraction, gave negative PCR results in the second extraction performed four months later, illustrating the frailty of RNA when faecal samples undergo several thaw and freeze procedures. In addition, samples belonging to the same individuals, but collected at different time points did not always provide consistent results, i.e., among the 12 individuals which samples were available in duplicate, 10 were initially negative and reconfirmed to be negative in the corresponding additional 10 samples. For the two remaining individuals the first samples were positive, but their duplicates were not. Three individuals were represented by three samples each: 2 were confirmed all three times to be negative, but the third individual was positive one out of three times.

Table 6.2: Characteristics of SIVwrc positive samples

| Sample n° | Age[a] | Sex[b] | Social Group | *pol* | *env*[c] |
|---|---|---|---|---|---|
| **32** | **A** | **nd** | **Bad1b** | **-** | **+** |
| **52** | **A** | **M** | **Bad1b** | **+** | **+** |
| **196** | **A** | **nd** | **Bad1b** | **+** | **+** |
| **223** | **A** | **M** | **Bad1b** | **+** | **+** |
| **268** | **A** | **F** | **Bad1b** | **-** | **+** |
| **280** | **A** | **M** | **Bad1b** | **-** | **+** |
| **296** | **A** | **M** | **Bad1b** | **-** | **+** |
| **299** | **A** | **M** | **Bad1b** | **+** | **+** |
| **110** | **A** | **nd** | **Bad2a** | **+** | **+** |
| **112** | **A** | **nd** | **Bad2a** | **+** | **+** |
| **115** | **A** | **M** | **Bad2a** | **+** | **-** |
| **116** | **A** | **M** | **Bad2a** | **+** | **+** |
| **175** | **A** | **F** | **Bad2a** | **-** | **+** |
| **237** | **A** | **M** | **Bad2a** | **+** | **+** |

[a]Age group at the time of sample collection: A, adult
[b]M, male; F, female
[c]Fragments PCR amplified

**Genetic diversity of SIVwrc in the two Taï red colobus communities**

To determine the relationships between the newly identified SIVwrc viruses to one another as well as to previously characterised SIVwrc strains from the Taï Forest, we constructed phylogenetic trees from partial *pol* and *env* sequences using a Bayesian inference approach. The previously published SIVwrc sequences obtained from four red colobus samples and the SIVolc sequence from one olive colobus collected during the Ebola study conducted in Taï National Park during the late nineties [77] were also included in this analysis. Samples SIVwrc-97CI-12, SIVwrc-96CI-M001 and SIVwrc-97CI-14 were collected in a region north of our study area, SIVwrc-00CI-04 was collected in the vicinity of our study groups and SIVwrc-98CI-04 was collected in the home range of our study groups (Figure 6.2). Corresponding SIV sequences from the SIVlho lineage (SIVmnd-1, SIVsun and SIVlho) were included in the analysis to serve as outgroups. Although we could not obtain *pol* and *env* fragments for every sample, the phylogenies yielded some intriguing results (Figure 6.3a and 6.3b).

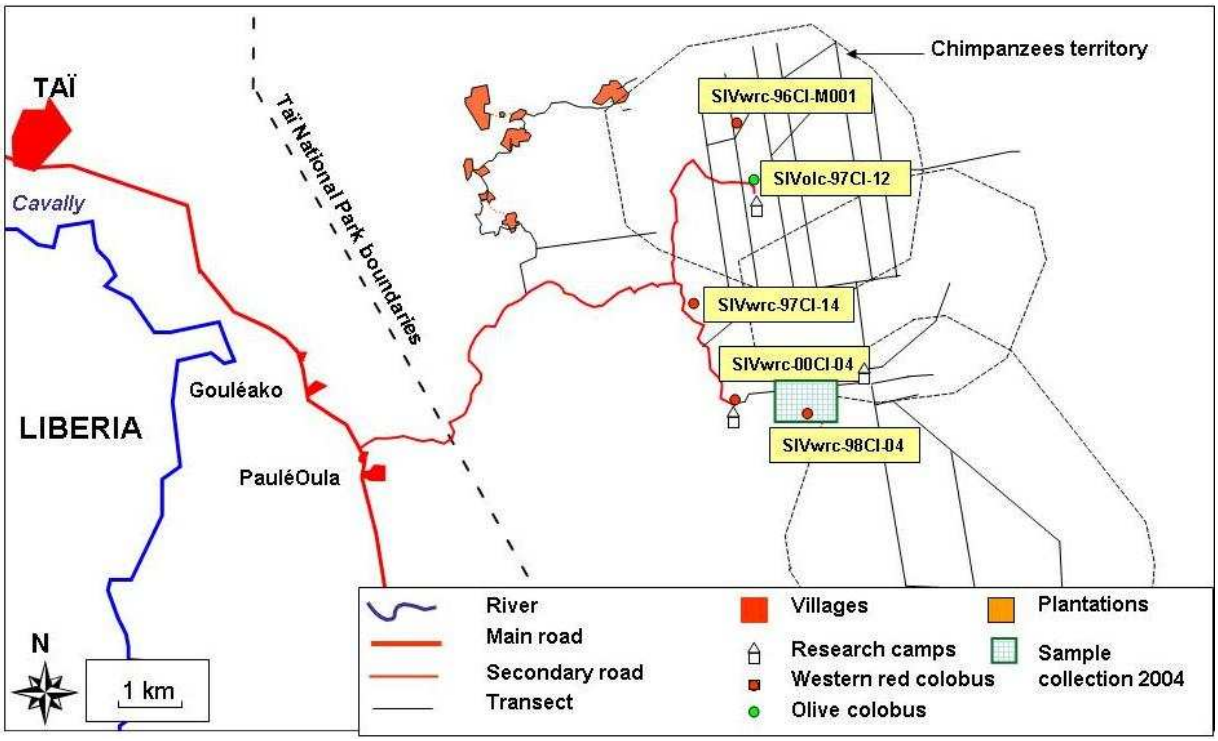
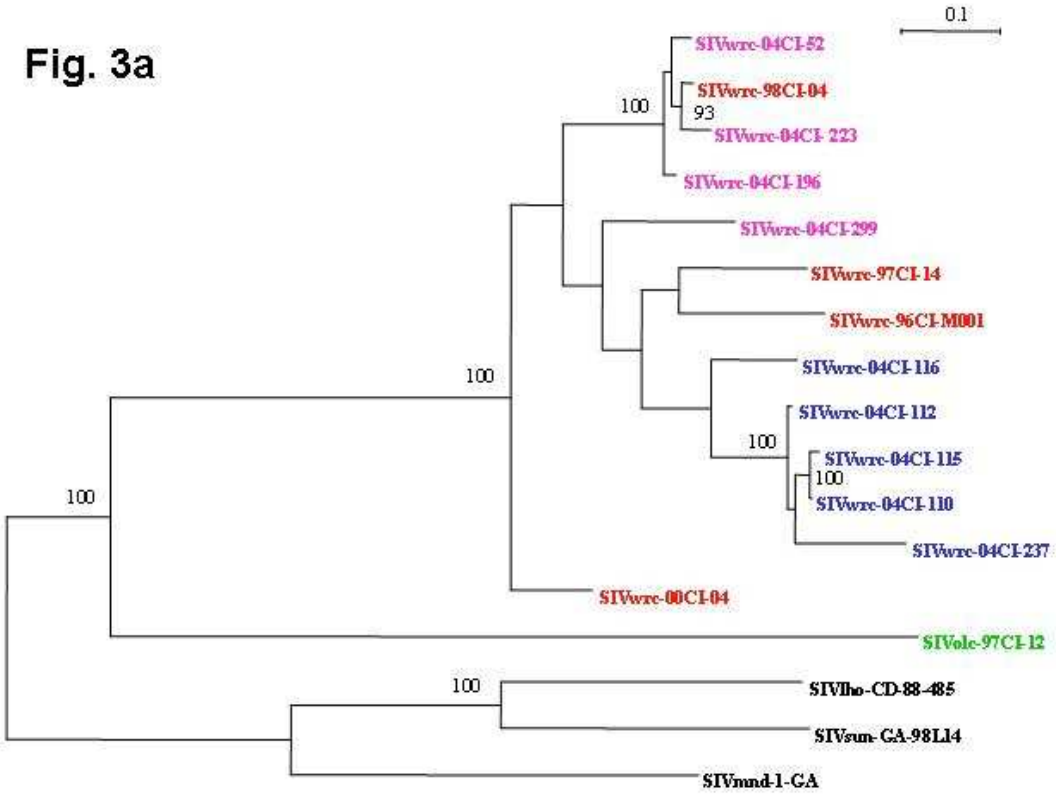



Figure 6.2: Map of the territory of the Taï Chimpanzees and Monkey project research sites

The proximity with crop plantations and villages is shown. Main and secondary roads as well as transects are depicted. Ranges of three habituated communities of chimpanzees are listed as well as the location of camp sites. Faecal samples collected between March and July 2004 are confined in the area delimited in green. The additional samples previously collected and included in the phylogenetic analysis [77] are marked with their respective numbers and with a red or a green dot for red and olive colobus samples respectively (courtesy of P. Formenty).

Previously as well as newly collected red colobus SIVs clustered together and formed a “SIVwrc” monophyletic group. Overall, within that group, we could observe two main well supported clades, each consisting of virus strains belonging to individuals coming from the same social group in both *pol* and *env* phylogenetic trees. Thus, all strains but one (*pol* phylogeny) or two (*env* phylogeny) fell into either Bad2a or Bad1b clusters. SIVwrc-04CI-116 did not group with the other Bad2a samples in both *pol* and *env* phylogenies. Its grouping within the Bad2a cluster in *pol* phylogeny was not supported at a significant level (posterior probability <91%; [423] whereas its basal position within the Bad1b cluster in the *env* tree was. Strain SIVwrc-04CI-116 thus appears to be more closely related to Bad1b samples than to Bad2a.

Similarly, SIVwrc-04CI-299 and SIVwrc-04CI-296 (Bad1b) branched independently from their group of origin (for SIVwrc-04CI-296, only the *env* fragment could be amplified). As observed in the phylogenetic tree, the genetic distances between unclustered taxa (SIVwrc-04CI-116 and SIVwrc-04CI-299) and Bad1b and Bad2a groups as well as between them were much higher than intragroup distances for Bad1b and Bad2a and were in the range of the distances between these two groups in the case of *pol* (Table 6.3a). For *env*, the average distance between SIVwrc-04CI-116 and Bad1b cluster was slightly higher than intragroup diversity for Bad1b or Bad2a and smaller than distances between any other groups (Table 6.3b). Interestingly, SIVwrc-98CI-04, from an animal collected near our study grid, also clustered with the SIVwrc strains of Bad1b in both *pol* and *env* phylogenies. In turn, SIVwrc-00CI-04, collected in close vicinity to the study area, branched independently from the two main groups in both *pol* and *env* phylogenies, as did SIVwrc-96CI-M001 and SIVwrc-97CI-14 collected about 2 and 4 km north of our study site respectively.



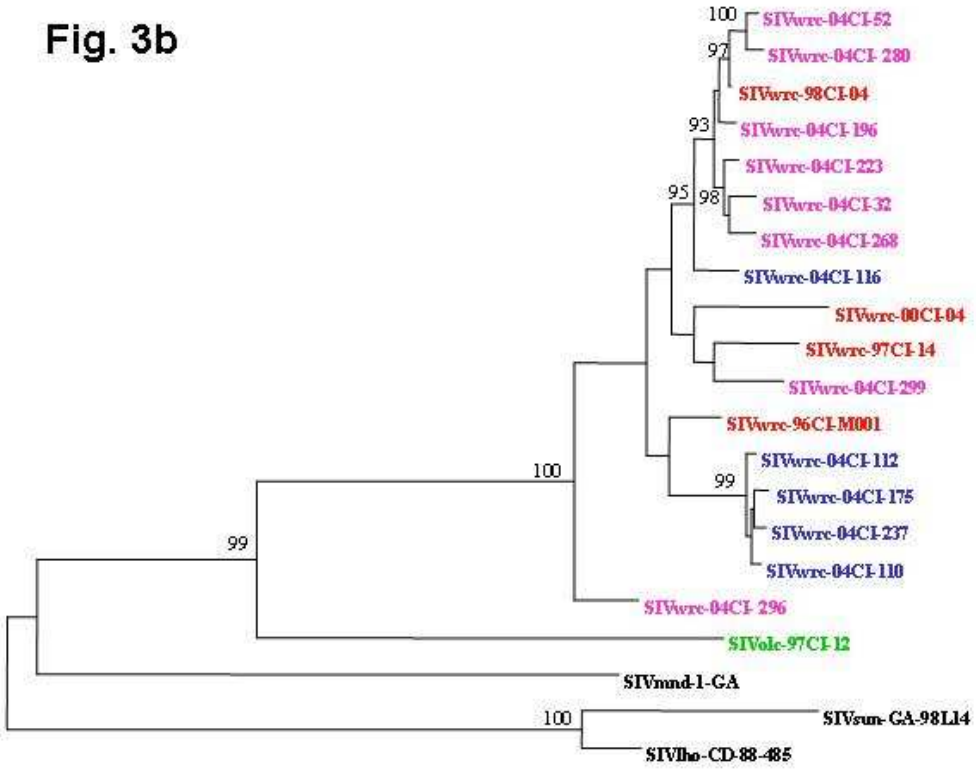




Figure 6.3: Phylogenetic relationship of SIVwrc positive samples in *pol* and *env* regions

Figure 6.3 (continued): Phylogenetic analysis of partial *pol* (polymerase) (a) and *env* (gp41, envelope transmembrane protein) (b). Sequences of the newly identified SIVwrc strains are highlighted by colours reflecting their social group, samples depicted in pink represent individuals belonging to group Bad1b, samples in blue belong to group Bad2a. Previously published SIVwrc and SIVolc strains collected during the Ebola study conducted in the Tai Forest (1997-2000) are depicted in red for red colobus and in green for olive colobus. The trees were inferred by the Bayesian method. Numbers on branches are posterior percentage probabilities (only values above the significance level of 91% are shown [423]). The scale bars indicate 0.1 substitutions per site.

Table 6.3: Within (in bold and italic) and between group distances among SIVwrc *pol* (a) and *env* (b) sequences

(a)

| *pol* | Bad1b | 299 | 116 | Bad2a |
|---|---|---|---|---|
| Bad1b | ***0.0411*** | | | |
| 299 | 0.1695 | **-** | | |
| 116 | 0.1864 | 0.1741 | **-** | |
| Bad2a | 0.1921 | 0.1737 | 0.1406 | ***0.0601*** |

(b)

| *env* | Bad1b | 299 | 296 | 116 | Bad2a |
|---|---|---|---|---|---|
| Bad1b | ***0.0427*** | | | | |
| 299 | 0.1695 | **-** | | | |
| 296 | 0.1369 | 0.1326 | **-** | | |
| 116 | 0.0699 | 0.1095 | 0.1221 | - | |
| Bad2a | 0.1125 | 0.1201 | 0.1369 | 0.1158 | ***0.0193*** |

## 6.5 Discussion

Although it is already known that western red colobus (*Piliocolobus badius badius)* from the Taï Forest in Côte d'Ivoire, are infected with SIV, this is the first time that a study has been conducted to estimate the extent of infection in a wild living population. We extracted RNA from faecal samples and we amplified fragments corresponding to portions of the *pol* and the *env* regions of the SIVwrc genome in 14 out of 53 adult individuals belonging to two social groups.

These results revealed a minimal infection level of 26% in these groups and showed that the majority of the new SIVwrc strains seemed to be specific of each red colobus group.

Based on the studies carried out on wild chimpanzee and gorilla populations, we initially tested western red colobus faecal samples for the presence of SIV infection using serological methods [183, 386]. Despite the fact that the first SIVwrc strains had been detected in blood by cross-reactive antibodies on INNO-LIA HIV tests, IgGs were absent or present only in too low quantities in Western red colobus faecal samples [77]. In general, a lower concentration of IgGs is expected when analysing urine or stools compared to whole blood [105, 294]. However, it cannot be excluded that antibody titers in faeces could also vary according to the host species investigated and could be related to different digestive physiology, diet composition and notably, the phytochemical composition of the leaves ingested. Colobines are mainly folivourous species and have enlarged sacculated forestomachs for microbial fermentation [269]. Consequently, all samples were tested for viral RNA, and we were able to amplify SIVwrc *pol* and/or *env* sequences in 14 (26%) out of 53 animals. To date, no study on red colobus monkeys has compared faecal and blood samples belonging to the same individuals. However, if we consider that 5 out of 10 red colobus blood samples were SIV positive in a previous study [77], that SIVsmm virion RNA detection in sooty mangabeys faecal samples revealed a 50% decreased sensitivity compared to the corresponding blood samples [217] and that SIV fragments amplifications are sometimes difficult to obtain from RNA extracted from faecal samples (93 % and 64% in *env* and *pol* fragments respectively in this study), we estimate that the percentage of SIV infection in the adult population living in Taï National Park should be at least 26% and could possibly reach 50%. Therefore, we conclude that wild-living red colobus monkeys represent a substantial reservoir of SIVwrc.

The relatively high SIV prevalence could partly be explained by the promiscuous social system in which red colobus monkeys live [196]. With the exception of a few cases, the grouping of the SIVwrc (Taï) viruses identified in both *pol* and *env* phylogenies, paralleled the host distribution into two social groups. This suggests that the infections within either group are epidemiologically linked. Long term behavioural

studies reported that red colobus monkeys live in large multi-male groups, with adult males having a lifelong breeding tenure in the group and male immigration being extremely rare. Dispersal is strongly female-biased and occurs when females reach the sub-adult stage of life, therefore before entering sexual maturity. Accordingly, extra group copulations have rarely been observed [196]. This could explain the close relationship between the SIVwrc viruses harboured by the two distinct social groups in our study. In order to make sure that the faecal samples containing near identical SIVwrc sequences were not inadvertently collected from the same individual, but were instead derived from horizontal or vertical transmission, we relied on observational data, repeated microsatellite testing and SIV PCR results to confirm the different origins of the positive samples as well as the absence of contaminations by closely related strains from individuals of the same group.

However, routes of SIV transmission are extremely challenging to determine, especially when observing an almost exclusively arboreal primate species, in comparison to ground dwelling primates which can be more easily monitored and where juveniles and infants can be better identified and ascribed to their respective mothers and their faecal samples more confidently detected. Most of the newly identified SIVwrc viruses fell into two clusters corresponding to the social group of origin of their host. Furthermore, SIVwrc-98CI-04, collected in 1998 within the range area of our two 2004 study groups, groups with the Bad1b viruses, thus supporting the hypothesis that those viruses parallel the host distribution. SIV infection has also been studied in wild living mangabeys from the Taï Forest, and nearly identical SIVsmm sequences have been reported in both related and unrelated individuals belonging to a single community suggesting vertical as well as horizontal routes of SIV transmission. However, highly divergent as well as recombinant virus strains were also identified, supporting the notion that the natural history of SIVsmm is one of a virus spread both within and between wild communities [322]. The fact that, unlike red colobus females, sooty mangabey females have stable dominance ranks [306], that there is a statistically significant association between high rank and faecal viral RNA positivity and that males are the gender which disperses (possibly to more than one group in a lifetime), could account for a higher virus divergence compared to that observed in the two red colobus communities under

study. In our study, a few SIVwrc viruses had intermediary positions between the two major groups in our phylogenies. SIVwrc-04CI-296 and SIVwrc-04CI-299 branch independently from their group of origin. Groups Bad1b and Bad2a are of course surrounded by other non habituated red colobus groups and these individuals could have been infected with SIV during agonistic behaviours with individuals from a neighbouring group. An interesting case is that of SIVwrc-04CI-116, which branches in the *pol* phylogeny apart from the SIV strains from the social group to which it belongs, and is closer to strains from the group Bad1b in the *env* phylogeny. Several hypotheses can be formulated *a posteriori* regarding the reasons for these results. Individual 116 could have been infected by getting wounded in a fight with males from the neighbouring group Bad1b or by a recently emigrated female, who had already contracted the infection in her natal group by vertical transmission. Our long term field studies identified SIVwrc-04CI- 116 to belong to an old male named ‘Adam’. In 1992 the territory under study was inhabited by two groups of about 90 individuals each, Bad1 and Bad2, which were followed until 1999. Between 1994 and 1998, each group started to split up into two sister fractions [389], that shared the same home range for 2-3 years during and after the splitting process. The smaller sister fractions slowly moved out of the main home range area and groups Bad 1b and Bad 2a became the major targets of observation [191]. Adam was observed moving between the two groups during the period when the fission of the larger Bad1 and Bad2 groups occurred. At the beginning of the fission process males moved between groups; the younger males were never observed in their non-natal sister group, but the older males were often going between groups until 1997-1998. Moreover, when well established in the Bad2a group, Adam was observed to suffer from impotency (A.H. Korstjens, personal communication) and therefore would have been unable to pass on his SIV infection, and indeed, among the individuals tested in the group Bad2a, none were identified with a closely related SIVwrc strain. If Adam was still a sexually active individual, we can speculate that individuals expressing double infections or a recombinant virus from the two neighbouring groups would have been observed. Nevertheless, data from more individuals, in particular females, would be required to confirm such a hypothesis.

Chimpanzees of the Taï Forest are strongly specialised in hunting colobus monkeys. It has been shown that the annual killing rate of colobus is 125 in 250 hunts, where 80% of kills are represented by red colobus and 13% by black & white colobus monkeys [31]. In light of the frequency of Taï chimpanzees predation on red colobus and considering the significant SIVwrc prevalence, we cannot exclude that this subspecies of chimpanzees might have been as well infected by SIV, which could have resulted in a low level of SIVwrc infection or the emergence of a recombinant SIV strain so far undetected by the current tools available. Moreover, the data obtained on STLV type 1 viruses isolated from chimpanzees in the Taï Forest evokes the potential for interspecies transmission of retroviruses through predatory relationships. Notably, one of the chimpanzee's isolates clustered with the STLV-1 isolated from the red colobus monkey 1497 [208, 209].

Chimpanzees do not represent the only threat for red colobus monkeys. Red colobus monkeys are also one of the most frequent catch of bushmeat hunters because they are found in large numbers and are easily detected by their loud vocalisations. Between April 1998 and March 1999, 2351 monkey carcasses were sold around the Taï National Park and of those 28% were red colobus [54]. Today, civil war and political instability also contributes to increasing poaching pressure. Additionally, SIVwrc has been detected in the same territory where sooty mangabeys have been found to harbour SIVsmm variants which are the ancestors of different groups of HIV-2, including those playing a major role in HIV-2 epidemic in West Africa. Given the significant frequency of SIVwrc infection in the wild, the relative abundance of red colobus, their cohabitation with other monkey species carrying genetically different SIVs and given the relatively high handling and consumption of their meat by chimpanzees and by the human population, there are many prerequisites for potential cross-species transmissions. In order better to document SIVwrc and their evolution among the *Colobinae* subfamily, full genomes need to be characterised among individuals representative of the genus *Piliocolobus* in West, Central and East Africa and subsequently compared to SIVs isolated from *Procolobus verus* as well as *Colobus* species across Africa. A thorough study of the SIVwrc genome could provide us with new tools for the search of SIV in *Pan troglodytes verus*. In the Taï Forest, screening

should be extended not only to chimpanzees, but also to other sympatric species, such as *Colobus polykomos* and especially to species associating with red colobus, e.g. *Cercopithecus diana*.

## 6.6 Acknowledgements

We thank the 'Ministère d'Enseignement Supérieur et Recherche Scientifique', the 'Ministère d'Agriculture et Resources Animales', the 'Centre Suisse de Recherche Scientifiques'(CSRS) in Abidjan, the 'P.A.C.P.N.T.' and the 'Centre de Recherche en Ecologie' in Côte d'Ivoire for support and permission to conduct research in the Taï National Park. We thank the Taï Monkey Project and in particular Ferdinand Bélé, Cécile Benetton and Bertin Diero, for helping out in samples collection; Christelle Butel, Fran Van Heuverswyn and Nicole Vidal for technical advice in the laboratory in Montpellier; Fabian Leendertz and Johannes Refisch, for involvement in the early phase of the project, Marcel Tanner and Jakob Zinsstag for critical support outside the field. The virology section of this study was financially supported by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de Recherches pour le SIDA (ANRS).

Sabrina Locatelli was supported by grants from the Commission for Research Partnerships with Developing Countries (KFPE), Bern to conduct field research and by the Messerli foundation**,** Zürich and the Guggenheim-Schnurr Foundation, Basel, Switzerland to conduct laboratory analysis on the monkey genetics.

# 7. Full molecular characterisation of a simian immunodeficiency virus, SIVwrc-*pbt* from Temminck's red colobus (*Piliocolobus badius temminckii*) from Abuko Nature Reserve, The Gambia

Sabrina Locatelli[1], Bénédicte Lafay[2], Florian Liegeois[1], Nelson Ting[3,4], Eric Delaporte[1], Martine Peeters[1*]

[1]UMR 145, Institut de Recherche pour le Développement (IRD), and University of Montpellier 1, Montpellier, France

[2]UMR CNRS-IRD 2724, Montpellier, France

[3]Anthropology Program, City University of New York Graduate Center, 365 Fifth Avenue, New York, NY 10016, USA

[4]New York Consortium in Evolutionary Primatology (NYCEP), USA

*Corresponding author and address:

Martine Peeters

UMR 145, Institut de Recherche pour le Développement (IRD)

911 Avenue Agropolis, 34394 Montpellier, France.

Phone : (+33) 0467416297

Fax : (+33) 0467416146

E-mail : martine. peeters@mpl.ird.fr

**This paper has been published in *Virology* (Epub ahead of print)**

## 7.1 Abstract

Simian immunodeficiency viruses (SIVs) are found in an extensive number of African primates, and humans continue to be exposed to these viruses by hunting and handling of primate bushmeat. The purpose of our study was to examine to what extent *Piliocolobus badius* subspecies are infected with SIV in order to better characterize SIVwrc in general and to gain further insight into the impact of geographic barriers and subspeciation on the evolution of SIVwrc. We analysed sixteen faecal samples and two tissue samples of the *P.b.temminckii* subspecies collected in the Abuko Nature Reserve (The Gambia, West Africa). SIV infection could only be identified in one tissue sample, and phylogenetic tree analyses of partial *pol* and *env* sequences showed that the new SIVwrc-*Pbt* virus is closely related to SIVwrc-*Pbb* strains from *P.b.badius* in the Taï Forest (Côte d'Ivoire), thus suggesting that geographically separated subspecies are infected with a closely related virus. Molecular characterization and phylogenetic analysis of the full-length genome sequence confirmed that SIVwrc-*Pbt* is a species-specific SIV lineage, although it is distantly related to the SIVlho and SIVsun lineages across its entire genome. Characterization of additional SIVwrc viruses is needed to understand the ancestral phylogenetic relation to SIVs from l'Hoest and sun-tailed monkeys and whether recombination occurred between ancestors of the SIVwrc and SIVlho/sun lineages.

**Keywords:** Red colobus, SIVwrc, evolution, genetic diversity, cross-species transmission

## 7.2 Introduction

It is now well established that the human immunodeficiency viruses, HIV-1 and HIV-2, are the results of cross-species transmissions of simian immunodeficiency viruses (SIV) naturally infecting non-human primates in sub-Saharan Africa. SIVsmm from sooty mangabeys (*Cercocebus atys atys*) is recognised as the progenitor of HIV-2, whereas SIVcpz from chimpanzees (*Pan troglodytes troglodytes*) and SIVgor from gorillas (*Gorilla gorilla gorilla*) in West-central Africa are the ancestors of HIV-1 [122, 183, 386]. Serological evidence of SIV infection has been shown for at least 39 of the 69 different primate species in Africa and has been confirmed by sequence analysis in 32. Complete SIV genome sequences are available for 19 species [142, 382]. Interestingly, only Old World primates are infected with SIVs, and only those from the African continent. Although SIVs are called immunodeficiency viruses, they generally do not induce an AIDS-like disease in their natural hosts, suggesting that they have been associated and evolved with their hosts over an extended period of time. However, recent reports have demonstrated that SIV infection in natural hosts can eventually lead to immunodeficiency, but this seems to occur only when animals have been infected over long periods of time. [216, 283].

Phylogenetic analyses have revealed high levels of genetic diversity among the known SIVs, but generally each primate species is infected with a species-specific virus. Although for some primates, virus and host phylogenies seem to match, cross-species transmission followed by recombination in distantly related primate species has been frequently documented [27, 168]. The most remarkable example is that of SIVcpz from chimpanzees, which is the result of the recombination between the ancestors of SIVs infecting red capped mangabeys (*Cercocebus torquatus*) and the ancestor of a clade of viruses (SIVgsn, SIVmon, and SIVmus) found in three *Cercopithecus* species, greater spot-nosed (*C.nictitans*), mona (*C.mona*), and moustached monkeys (*C.cephus*), all of which are hunted by chimpanzees in West-Central Africa [14, 242]. A single species can also be infected with different viruses. For example, mandrills (*Mandrillus sphinx*) living in Gabon south of the Ogoué river are infected with SIVmnd-1, but those spanning the regions of southern Cameroon and northern Gabon are infected with SIVmnd-2 [276, 350, 370]. Even in the absence of rivers as geographical barriers, two viruses can co-

circulate in one species within small geographic areas, as observed in moustached monkeys from Cameroon infected with SIVmus-1 and SIVmus-2 [1].

SIV transmission from primates to humans is most likely the result of contact with infected blood and tissues from primates hunted for bushmeat [143]. The ancestors of HIV-1 and HIV-2 have crossed the species barrier to humans on multiple occasions, but the transmission potential of the other primate lentiviruses remains unknown [143]. However, we have shown that a substantial proportion of wild-living monkeys in Cameroon are infected with SIV and that humans are exposed to a plethora of genetically diverse viruses through hunting and handling of bushmeat [287], thus emphasizing the need to continue surveillance and characterization of SIVs in primates. With the exception of SIVcpz and SIVgor, all SIVs identified so far originate from primates belonging to the family Cercopithecidae, or Old World monkeys, which is subdivided into two subfamilies: the Colobinae and Cercopithecinae [93, 139]. Due to their high species number and large distribution in sub-Saharan Africa, the Cercopithecini tribe of the Cercopithecinae represents today the largest reservoir for SIV. The African Colobinae instead are composed of only about 11 species which are classified in three genera, *Colobus*, *Procolobus* and *Piliocolobus* [139, 142]. Their geographic distribution spans the regions in Africa where equatorial forests are still present, ranging from The Gambia in the west to Zanzibar in the east. In certain regions of Africa, colobines are heavily hunted by the human population [54] and also, by chimpanzees [31, 352, 356, 368], possibly exposing the human and the chimpanzee populations to additional retroviruses. SIV infection has been identified in at least one species of each African colobine genus, but only SIVcol, from a mantled guereza (*Colobus guereza*) in Cameroon has been fully characterized [77, 78]. Whereas SIVcol forms a separate divergent lineage, molecular characterization of a 2000 bp fragment in *pol* showed that SIVwrc from western red colobus (*Piliocolobus badius badius*) and SIVolc from olive colobus (*Procolobus verus*) in the Taï Forest, Côte d'Ivoire each forms a species-specific lineage unrelated to SIVcol [77]. The *Piliocolobus badius* species can be further subdivided into three geographically isolated subspecies: *P.b.badius*, in Guinea, Sierra Leone, Liberia and Côte d'Ivoire; *P.b.waldroni*, formerly found in Ghana and Côte d'Ivoire east of the Bandama river, but nearly extinct today [236] and

*P.b.temminckii* which is found in Senegal, The Gambia, Guinea Bissau and NW Guinea.

The purpose of our study was to examine to what extent other *Piliocolobus badius* subspecies are infected with SIV in order to better characterize SIVwrc in general and to gain further insight on the impact of geographic barriers on the evolution of SIVwrc. We analysed sixteen faecal samples and two tissue samples of the *P.b.temminckii* subspecies collected in the Abuko Nature Reserve in The Gambia, West Africa. SIV infection could only be identified in one tissue sample, and the new SIVwrc*Pbt* virus was closely related to SIVwrc*Pbb* strains from the Taï Forest in Côte d'Ivoire. Full genome sequence revealed that SIVwrc is a species-specific SIV lineage with a phylogenetic relation to SIVs from the l'Hoest lineage, thus suggesting an ancestral link of their respective genomes.

## 7.3 Materials and Methods

### Primate specimens and collection site

Sixteen faecal samples and two tissue samples from carcasses found on the forest floor were collected between January and February 2005 in the Abuko Nature Reserve, The Gambia. The Abuko Nature Reserve is situated outside the village of Lamin in the Kombo North District, 25km from Banjul (Figure 7.1a and 7.1b). The reserve was officially declared a nature reserve in 1968. The 106.6 ha reserve is a mosaic habitat consisting mostly of tree and shrub savannah (56.6%), woodland savannah (24.8%) and gallery forest (16%) [353]. About 200 red colobus live there in 5 groups [354]. Faecal samples were collected in the morning underneath sleeping trees from opposite ends of the red colobus range to ensure sampling from different individuals. Samples were between five minutes and two hours old at collection. They were put directly into RNA*later*® (1/1 vol) and were kept at room temperature for less than a week before storage at -20°C.

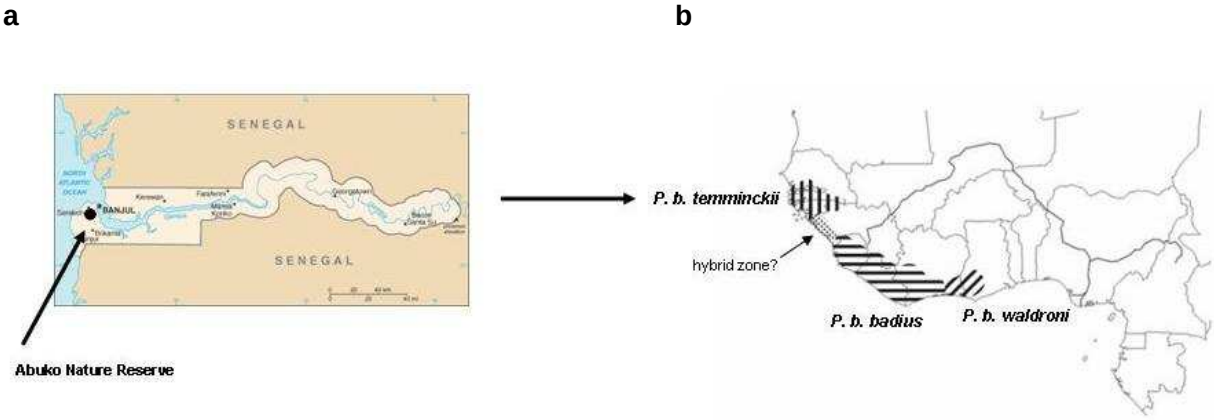



Figure 7.1: Location of the Abuko Nature Reserve, The Gambia, where the SIVwrc*Pbt*-05GM-X02 specimen was collected (a) and geographical distribution of the western *Piliocolobus* subspecies [140, 272] (b)

**Nucleic acid extraction from faecal and tissue samples**

Viral RNA was extracted from faecal samples using the RNAqueous-Midi kit (Ambion, Austin, Texas, USA) as previously described [183, 321]. Briefly, 6 ml of lysis-binding solution were added to 1.5 ml of faecal sample solution and vortexed vigorously until the sample was thoroughly homogenized. The suspension was clarified by centrifugation (at 4500 RPM for 5 min), and an equal volume of 64% ethanol was added. The solution was passed through a glass fibre filter unit to bind nucleic acids and washed three times with washing buffer. The nucleic acids were then eluted (1200 µl) and subsequently precipitated with LiCl and spun at 13000 RPM for 30 min. The resulting pellet was washed once with cold 70% ethanol, air dried, resuspended in 50 µl of RNase free-water and then stored at -80℃. Total DNA was extracted from liver tissue using the Qiagen Qiamp®DNA Micro Kit according to the manufacturer's instructions (Qiagen, Valencia, California, US); once extracted, RNA was stored at -80 and DNA at -20℃.

**Amplification of SIVwrc sequences from faecal RNA**

RT-PCR amplification of faecal virion was performed using two sets of primers specific for SIVwrc *pol* and *env* sequences as previously described [220] . cDNA was synthesized using the wrcpolR1/wrcenvR1 primers followed by nested PCRs using primers F1/R1 and F2/R2 as inner and outer primers, respectively. The *pol* primers included wrcpolF1 (5'-TAGGGACAGAAAGTATAGTAATHTGG-3') and wrcpolR1 (5'-GCCATWGCYAATGCTGTTTC-3') as outer primers and wrcpolF2 (5'AGAGAC AGTAAGGAAGGGAAAGCAGG-3') and wrcpolR2 (5'-GTTCWATTCCTAACCA CCAGCADA-3') as inner primers for the second PCR round. The *env* primers included wrcenvF1 (5'-TGGCAGTGGGACAAAAATATAAAC-3'), wrcenvR1 (5'-CTGGCAGTCCCTCTTCCAAGTTGT-3'), wrcenvF2 (5'-TGATAGGGMTGGCTCCT GGTGATG-3') and wrcenvR2 (5'-AATCCCCATTTYAACCAGTTCCA-3'). The amplified regions correspond to the 3' end of the *pol* gene and the gp41 region of the *env* gene. PCRs were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Manheim, Germany) under the conditions previously described [220]. Briefly, a hot start at 94℃ for 2 min was followed by 10 cycles of denaturation at 92℃ for 20 s, annealing at 45℃ for 45s, extension at 72℃ for 1.5 min, and 20 cycles with the annealing temperature increased to 50℃ with extension at 72℃ for

1.5 min. Amplification was completed by a final extension at 72℃ for 5 min. PCR conditions for the second PCR round were the same except that the extension time during cycling was 45s. RT-PCR products from *pol* (~650 bp) and *env* (~570 bp) regions were purified (Q-Biogene, Illkirch, France), and directly sequenced using the inner (F2/R2) primers on an ABI 3130xl Genetic Analyser (Applied Biosystem, Courtaboeuf, France). Sequences were then checked and assembled using the software package Lasergene (DNASTAR Inc. Madison, USA).

**PCR amplification and sequencing of full-length SIVwrc-*Pbt* genome**

In addition to the partial *pol* and *env* fragments, a small *gag* fragment was also amplified using degenerate consensus primers designed from the alignment of the gag region of two SIVwrc samples from *Piliocolobus badius badius* (SIVwrc-98CI-04 and SIVwrc- 97CI-14, collected in Côte d'Ivoire in the context of an Ebola study [77].

The complete SIVwrc genome was then obtained by amplifying overlapping PCR fragments of integrated genomic as well as unintegrated circular DNA using a combination of specific SIVwrc-05GM-X02 primers and consensus primers. More precisely, three fragments spanning the *gag-pol*, *pol-vif-env* and *env-nef*-LTR-*gag* regions were amplified and specific primers were designed to sequence these fragments. The primers used are shown in Table 7.1, and the corresponding amplification strategies are depicted in Figure 7.2. PCR amplifications were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Manheim, Germany) according to the manufacturer's instructions. Each amplification reaction included a manual hot-start at 94℃ followed by 35 cycles with a denaturation step at 94℃ for 20 sec, an annealing temperature set according to the primer melting temperatures and a variable extension time depending on the size of the expected fragment (1 min/kb). PCR products were purified on agarose gel and directly sequenced using cycle sequencing and dye terminator methodologies (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq FS DNA polymerase [PE Biosystems, Warrington, England]) on an automated sequencer (ABI 3130XL, Applied Biosystems, Courtaboeuf, France) using a primer walking approach. To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences using SEQMAN DNASTAR software (Lasergene, DNASTAR, Inc., Madison, WI).

Table 7.1: Primers used to amplify the genome of sample SIVwrc*Pbt*-05GM-X02

| PCR | Fragment[a] | Size (kb) | Primers |
|---|---|---|---|
| First round | A | ~ 0.25 | GAGwrcF1 (5'-ATD[b]GAGGATAGAGGNTTTGGAGC-3') |
| | | | GAGwrcR1 (5'-GCCCTCCTACTCCTTGACATGC-3') |
| Second round | | | GAGwrcF2 (5'-CCAACAGGGTCAGATATAGCAG-3') |
| | | | GAGwrcR2 (5'-ACTTCTGGGGCTCCTTGTTCTGCTC-3') |
| First round | B | ~ 0.67 | POLwrcolF1 ( 5'-TAGGGACAGAAAGTATAGTAATHTGG-3') |
| | | | POLwrcolR1 (5'-GCCATWGCYAA TGCTGTTTC-3') |
| Second round | | | POLwrcolF2 (5'AGAGACAGTAAGGAAGGGAAAGCAGG-3') |
| | | | POLwrcolR2 (5'-GTTCWATTCCTAACCACCAGCADA-3') |
| First round | C | ~ 0.58 | ENVwrcolF1 (5'-TGGC AGTGGGACAAAAATATAAAC-3') |
| | | | ENVwrcolR1 (5'-CTGGCAGTCCCTCTTCCA AGTT GT-3') |
| Second round | | | ENVwrcolF2 (5'TGATAGGGMTGGCTCCTGGTGATG3') |
| | | | ENVwrcolR2 (5'-AATCCCCATTTYAACCAGTTCCA-3') |
| First round | D | ~2.6 | GAGwrcF1 (5'-ATD[b]GAGGATAGAGGNTTTGGAGC-3') |
| | | | POLpbtR1(5'-GTATTTCCTCCTATCCCTTTATGTGCTG-3') |
| Second round | | | GAGwrcF2 (5'-CCAACAGGGTCAGATATAGCAG-3') |
| | | | POLpbtR2 (5'-AGGGAGATTCACTTTGAGTTGGGTG-3') |
| First round | E | ~ 3 | POLpbtF1 (5'-GCACCCACTTGGAAGGAAAAATCAT-3') |
| | | | ENVpbtR1 (5'-ACTGTTGATACCGTGCCCATTG-3') |
| Second round | | | POLpbtF2 (5'-CCTATCAAACAGCACTTTTCACCCT-3') |
| | | | ENVpbtR2 (5'-GCTCCTCGTTTTTCTCTATGATGGT-3') |
| First round | F | ~ 2.8 | GAGwrcR1 (5'-GCCCTCCTACTCCTTGACATGC-3') |
| | | | ENVpbtF1 (5'-CACCTGCTTGGAATAATGAAACA-3') |
| Second round | | | GAGwrcR2 (5'-ACTTCTGGGGCTCCTTGTTCTGCTC-3') |
| | | | ENVpbtF2 (5'-TAAGAACAGACACCTTGATGAGTAAT-3') |

[a]Letters correspond to the fragments labelled as such in Figure 7.2

[b]D= G or A

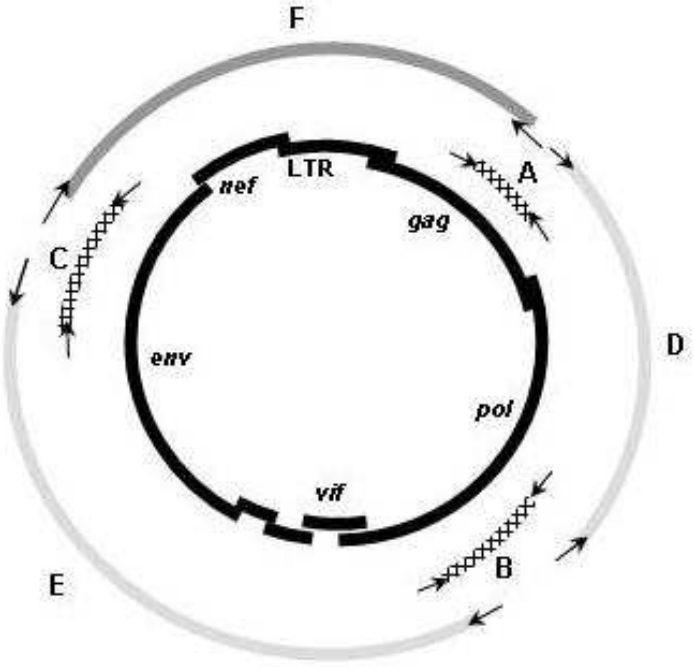



Figure 7.2: Schematic representation of the PCR amplification of full-length SIVwrc sequences from uncultured liver cells. The positions of the various amplification products are shown in relation to an unintegrated circular intermediate of SIVwrc*Pbt*-05GM-X02. Primer sets and fragment designations are identical to those in Table 7.1.

### Phylogenetic analysis of partial *pol* and *env* SIVwrc sequences

The new *pol* and *env* sequences were compared to previously published SIVwrc sequences identified in western red colobus from the Taï Forest [77, 220] . The model of evolution for *pol* and *env* (general time reversible model of evolution with a gamma distribution of substitution rates) was selected under the Akaike information criterion using Modeltest v3.7 [302]. Bayesian inferences were performed using MrBayes v3.1 [313]. The tree-space was explored using four chains over 30,000,000 generations sampled every 100 generations. The burn-in value was fixed at 10% of the total generation number after empirical determination of the convergence. Bayesian parameters were examined with the Tracer program (http://evolve.zoo.ox.ac.uk/software.html?id=tracer). *Pol* and *env* phylogenies of red colobus monkeys' SIV were rooted using sequences of corresponding regions in SIVlho from l'Hoest monkeys (*Cercopithecus lhoesti*) (AF188114), SIVsun from sun-tailed monkeys (*Cercopithecus solatus*) (AF131870) and SIVmnd-1 from mandrills

(*Mandrillus sphinx*) (M27470) as well as SIVolc from olive colobus (*Procolobus verus*) (accession and manuscript in preparation).

**Sequence and phylogenetic analyses of the full-length SIVwrc-05GM-X02 genome**

The new SIVwrc nucleotide sequence was compared to various previously published SIV sequences (see below). Predicted protein sequences were aligned using ClustalW [373]; where necessary, minor manual adjustments were performed using SEAVIEW [120]. Sites that could not be unambiguously aligned were excluded from the analyses. Proteome sequences were generated by joining deduced Gag, Pol, Vif, Env and Nef amino acid sequences; the carboxy terminal Gag, Pol and Env amino acid sequences that overlapped with Pol, Vif, and Nef amino acid sequences, respectively, were excluded. In order to study whether the newly characterized SIVwrc sequence was recombinant with any of the other SIV lineages, a similarity plot analysis was performed on the proteome sequence alignment with SIMPLOT package version 2.5 [221], using a sliding window of 200 amino acids (aa) moved in steps of 20 aa.

Phylogenies were inferred by the Bayesian method [419], implemented in MrBayes version 3.1 [313], run for 2,000,000 to 4,000,000 generations with a 10% burn-in. Parameters were examined with the Tracer program (http://evolve.zoo.ox.ac.uk/software.html/id=tracer). Four major regions of the proteome, Gag, Pol1, Pol2 and Env, were selected on the basis of the Simplot analysis. Using the mixed model in MrBayes indicated that the rtREV model of amino acid change [90] was most appropriate; this model was thus used with gamma distributed rates of substitution across sites.

**Secondary structure prediction**

The transactivation response element (TAR) secondary structure was predicted using the software GENEQUEST DNASTAR (Lasergene, Madison, US).

**Nucleotide sequence accession numbers**

The virus strain whose full length sequence was determined in this study can be found under the GenBank accession number AM937062.

## 7.4 Results

**SIV infection in faecal and tissue samples from Temminck's red colobus (*Piliocolobus badius temminckii*) in Abuko Nature Reserve, The Gambia**

Despite the positive results obtained in faecal samples from chimpanzees and gorillas [183, 385, 386], antibody detection in faecal samples from western red colobus was unsuccessful and samples were thus directly screened with molecular tools for SIV infection [220]. Sixteen faecal samples and two tissue samples collected in Abuko Nature Reserve, The Gambia, were analysed for SIV infection using SIVwrc specific primers, which amplified partial *pol* and *env* regions in samples from the *P.badius badius* subspecies collected in the Taï Forest, Côte d'Ivoire [220] (Figures 7.1a and 7.1b). None of the above mentioned fragments was amplified in the faecal samples, but in one out of two tissue samples 672 bp and 578 bp fragments in the *pol* and gp41 *env* regions, respectively, were amplified. This sample was named SIVwrc*Pbt*-05GM-X02, with *Pbt* referring to the *P.b.temminckii* subspecies (Table 7.1 and Figure 7.2).

**Phylogenetic analysis of partial *pol* and *env* sequences of the new SIVwrc*Pbt* isolate from Temminck's red colobus**

To determine the relationships between the newly identified SIVwrc*Pbt*-05GM-X02 in a Temminck's red colobus (*P.b.temminckii*) and previously characterised SIVwrc strains from the red colobus subspecies in Côte d'Ivoire (*P.b.badius*), we constructed phylogenetic trees from partial *pol* and *env* sequences using SIVolc and SIVs belonging to the SIVlho lineage (SIVmnd-1, SIVsun and SIVlho) as outgroups. In both phylogenies, SIVwrc*Pbt*-05GM-X02 falls within the SIVwrc*Pbb* cluster (Figure 7.3).

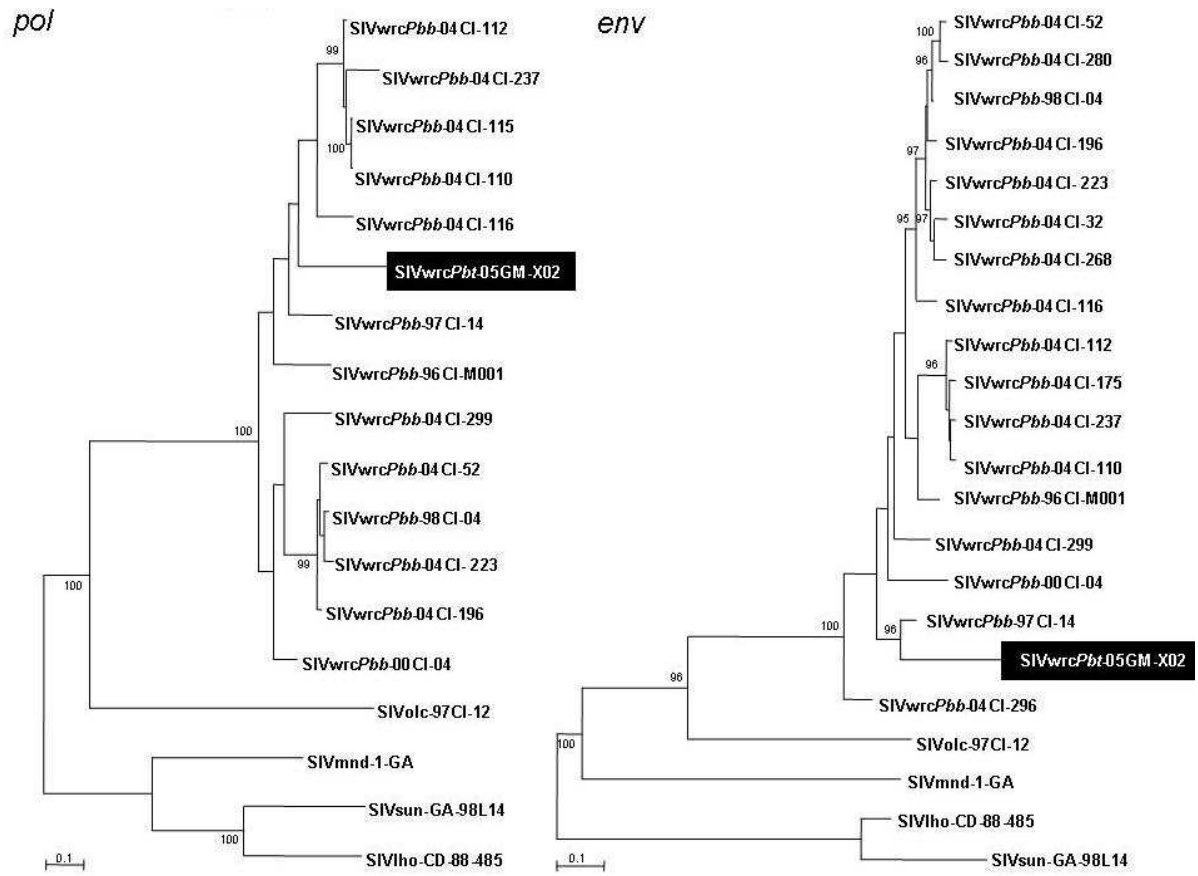



Figure 7.3: Phylogenetic analysis of partial (a) *pol* (polymerase,.~670 bp) and (b) *env* (gp41, envelope transmembrane protein, .~570 bp) genomic regions. The sequence of the newly identified SIVwrc*Pbt* strain is highlighted. Previously published SIVwrc*Pbb* and SIVolc strains collected during the Ebola study conducted in the Taï Forest (1997-2000) as well as SIVwrc*Pbb* strains from red colobus samples collected in 2004 are also included in the analysis [77, 219]. The trees were inferred by the Bayesian method. Numbers on branches are posterior percentage probabilities (only values above the significance level are shown). The scale bars indicate 0.1 substitutions per site.

## Full-length genome sequence and genomic organization of SIVwrc*Pbt*-05GM-X02

In order to ascertain this result and to better characterize the SIVwrc virus, we sequenced the full-length genome of the SIVwrc*Pbt*-05GM-X02 strain. Because of the apparent high degree of genetic similarity to SIVwrc*Pbb* in *pol* and *env*, we designed consensus primers in *gag* from an alignment of two unpublished SIVwrc*Pbb gag* sequences (SIVwrc*Pbb*-97CI-14 and SIVwrc*Pbb*-98CI-04) to amplify a 252 *gag* bp fragment for the new SIVwrc*Pbt*-05GM-X02 sample. Based on the partial sequences obtained in *gag* and the 3' end of the *pol* and gp41, strain specific primers were designed to amplify the remainder of the SIVwrc genome.

The complete genome sequence of SIVwrc*Pbt*-05GM-X02 was then obtained by successive nested PCRs as illustrated in Figure 7.2 and using primers shown in Table 7.1. Briefly, we proceeded to PCR amplifications spanning the regions of *gag-pol* (fragment D), *pol-env* (fragment E) and *env-nef*-LTR-*gag* for the circular unintegrated form of the virus (fragment F). These amplified fragments corresponding to amplicons of 2.6 kb, 3.0 kb, and 2.8 kb, respectively, were gel purified and sequenced with specific primers. The concatenated linear complete genome contains 8709 bp, including the LTR fragment. The SIVwrc genome was compared to the other primate lentiviruses and displayed the expected reading frames for *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *env* and *nef*. As for most of the SIV lineages, SIVwrc*Pbt*-05GM-X02 did not encode a *vpu* or *vpx* analogue. The genomic organisation of SIVwrc is thus similar to that of, e.g., SIVagm, SIVsyk, SIVmnd-1, SIVcol or the SIVlho lineage. The SIVwrc long terminal repeat (LTR) contained all the characteristic features of other primate lentivirus LTRs including TATA, 1 potential NF-kB site, and 1 potential SP-1 region (data not shown). The secondary structure prediction of the SIVwrc tar element showed an unusual organisation with two identical stem-loops consisting of 3 nucleotides bulges (GCC) and 7-bp stems with a 5-bp terminal loop(5'-UGGUC-3'; Figure 7.4).

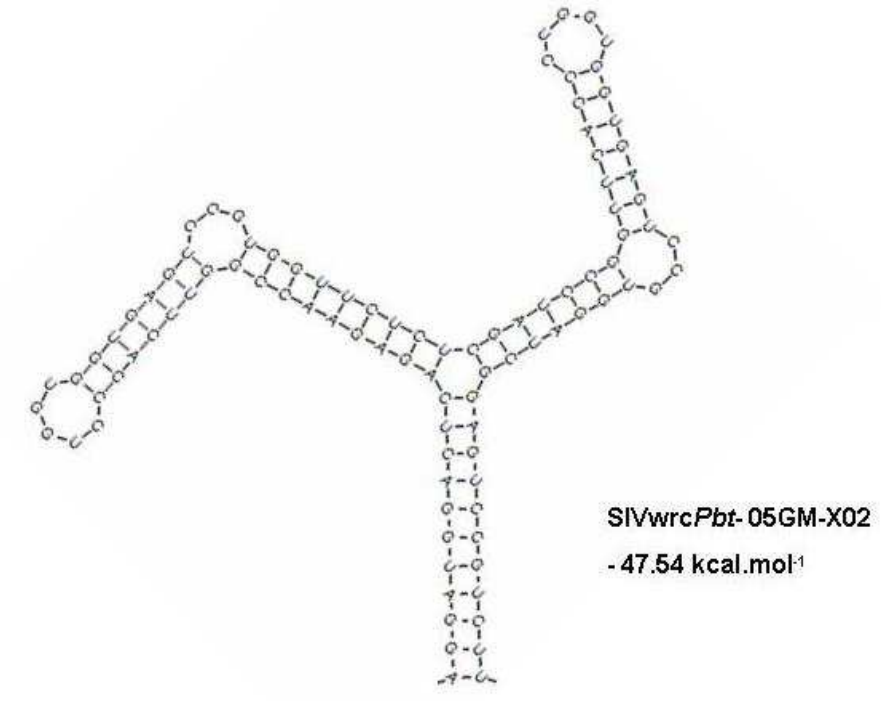



Figure 7.4: Secondary structure prediction of the SIVwrc*Pbt*-05GM-X02 TAR element with the lowest free energy value (-47.54 kcal.mol $^{-1}$)

**Phylogenetic relationship between SIVwrc*Pbt*-05GM-X02 and the other SIV lineages**.

To compare the SIVwrc*Pbt*-05GM-X02 genome to previously characterised SIV strains, we performed similarity plots of concatenated Gag, Pol, Vif, Env and Nef protein sequences. Figure 7.5a and 7.5b depict the trend of amino acid similarities between SIVwrc*Pbt*-05GM-X02 and representatives of SIV strains from the major lineages. In the Gag and the N-terminal part of Pol, SIVwrc*Pbt*-05GM-X02 seems to be equidistantly related to the other SIV lineages. Interestingly, in the Pol1 fragment, SIVwrc*Pbt*-05GM-X02 is closest to the SIVlho and SIVcol lineages (represented by strains SIVlho, SIVsun14, SIVmndGB1 and SIVcolCGU1). In the Env fragment, SIVwrc*Pbt*-05GM-X02 is close to the SIVlho, SIVmnd-1 and SIVmnd-2/drl lineages (strains SIVdrl1FAO, SIVmnd14cg, and SIVlho, SIVsun14, SIVmndGB1). In that latter part of the genome, the SIVwrc Simplot analysis identifies two groups of similarity that correspond to the SIVlho/SIVmnd-1/SIVmnd-2/drl and to all other SIVs, respectively (Figure 7.5).

We then constructed phylogenetic trees for Gag, Env as well as for two segments of Pol corresponding to the regions identified in the similarity analysis (Pol1 and Pol2) (Fig. 7.6). Overall, SIVwrc*Pbt*-05GM-X02 appears to be more closely related to SIVs from the SIVlho/sun and SIVmnd-1 lineages. The grouping of these taxa was supported by high posterior probabilities (above the 91% threshold; [423] in Gag, Pol1 and Env trees and at a slightly lower (non significant) value in the Pol2 phylogeny. SIVcol seems also to cluster with this group of viruses in the Gag, Pol1 and Env trees, although generally with higher degrees of divergence. Only in the Pol2 fragment does the SIVcol strain form a separate branch.

Interestingly, in accordance with the similarity plot data, the branching pattern of SIVwrc, SIVlho, SIVsun, SIVmnd-1 in the Env phylogeny (i.e. a long internal branch leading to shorter than expected ingroup branches relative to relationships among other taxa in the tree) suggest a more complex evolutionary history than for the rest of the genome in the SIVlho/sun/mnd-1/wrc lineages, possibly involving recombination.

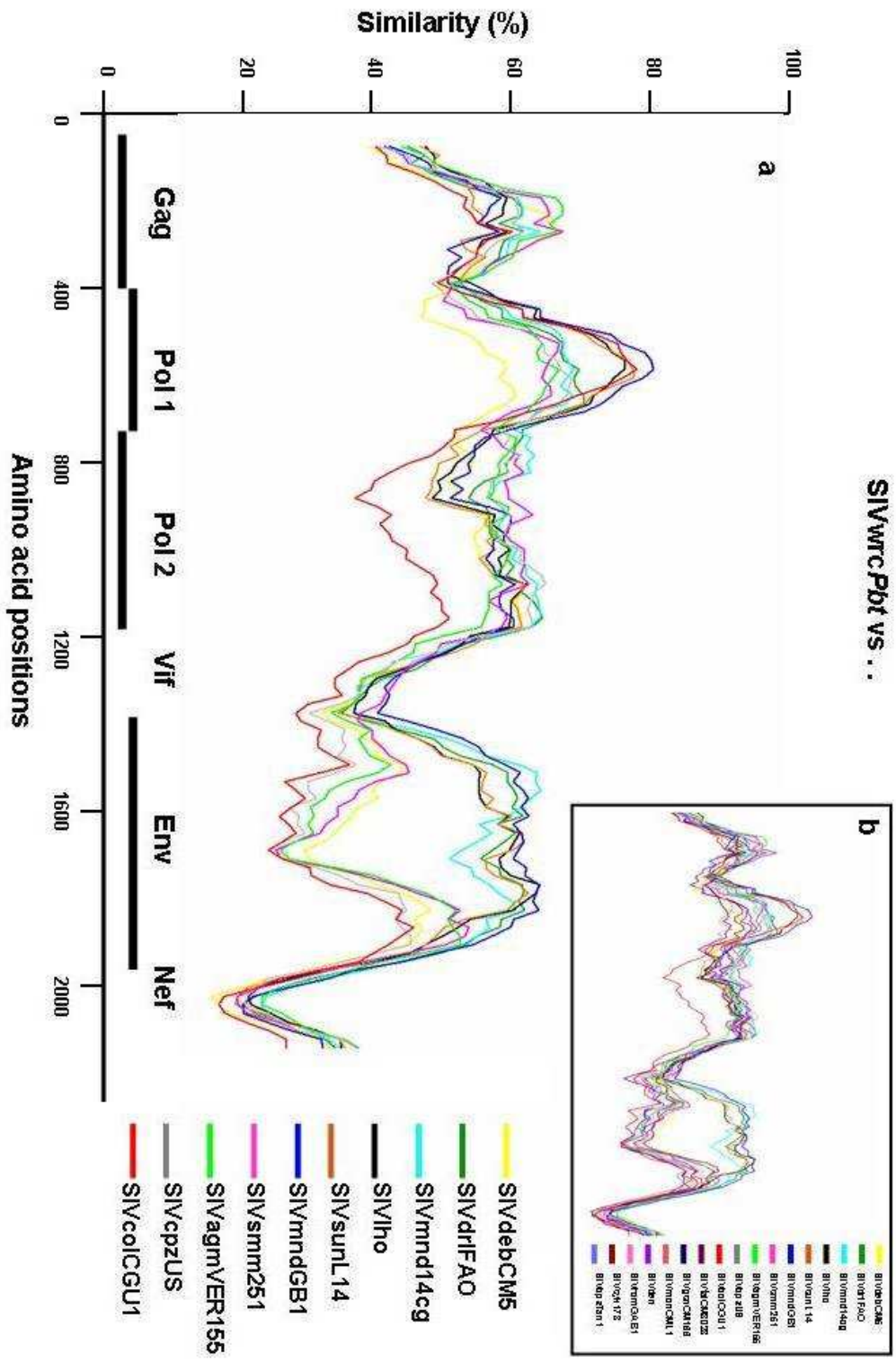



Figure 7.5: Similarity plots of concatenated Gag, Pol1 (corresponding to residues 1-436 of Pol in SIVwrc*Pbt*-05GM-X02), Pol2 (corresponding to residues 437-1013 of Pol in SIVwrc*Pbt*-05GM-X02), Vif, Env and Nef protein sequences showing the extent of genetic similarity between SIVwrc*Pbt*-05GM-X02 and 10 (a) versus 17 (b) strains from other SIV lineages. The proportion of amino acid sequence similarity per 200 residues window (vertical axis) is plotted against the midpoint of the sequence window (horizontal axis).

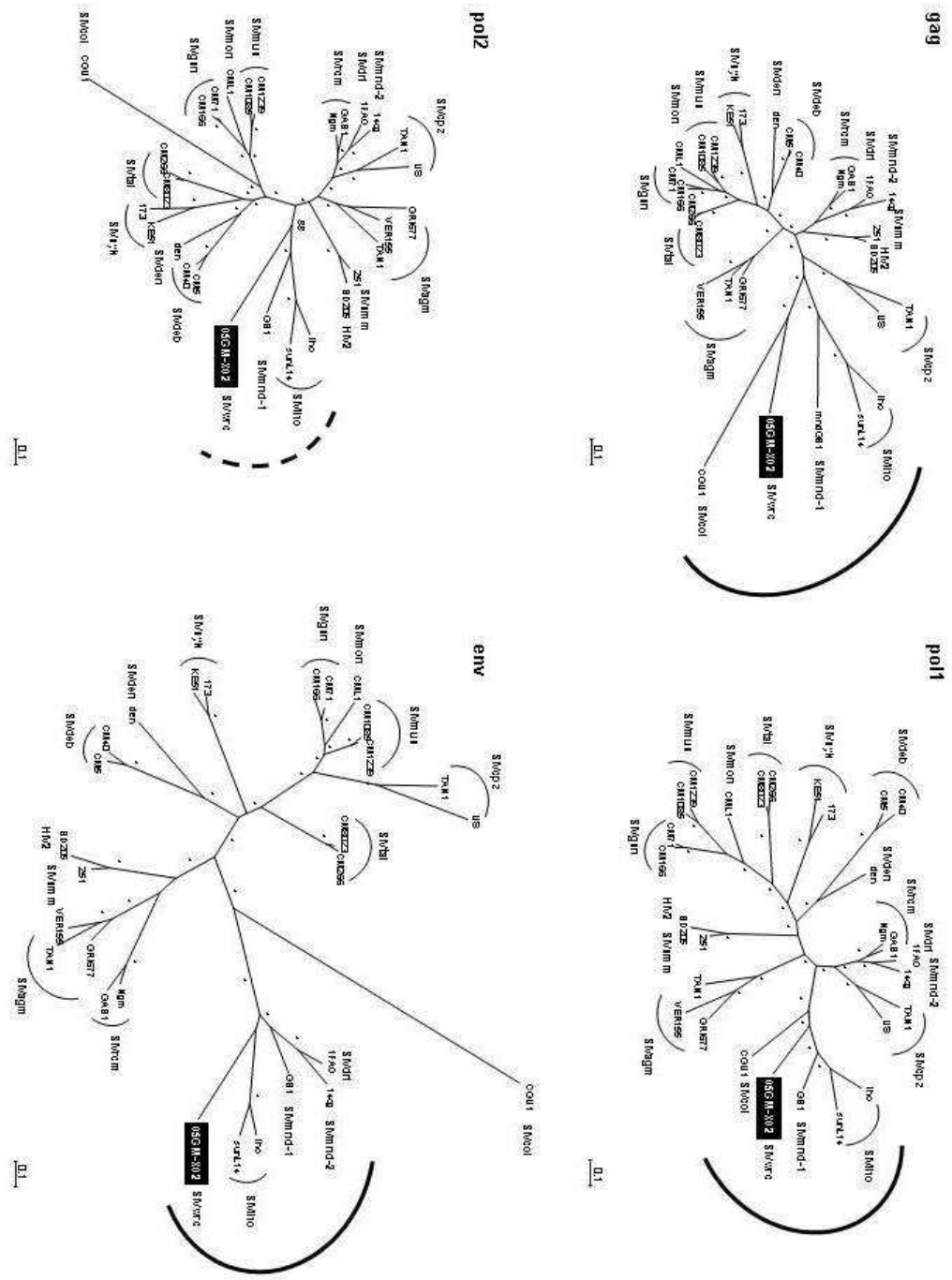



Figure 7.6: Phylogenetic relationships of the newly derived SIVwrc*Pbt* sequence to other SIV lineages. Trees were inferred from unambiguous parts of the protein sequence alignments from: (Gag, Pol1, Pol2 and Env. The newly characterized SIVwrc*Pbt* full-length genome is boxed. The numbers on nodes are estimated posterior probabilities, values greater than 92% are represented by an asterisk (*). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacements per site.

**Functional motifs in the SIVwrc genome**

Among the African primates the genus *Cercopithecus* harbours the largest number of species known to be infected with SIVs, and recently a *Cercopithecus*-specific SIV lineage has been described [27]. SIVs derived from different *Cercopithecus* species consistently form one highly supported group in phylogenetic trees despite examples of co-evolution between viruses and hosts and cross-species transmission within this genus. In addition, they share functional motifs in *gag* and *env* that distinguish them from other primate lentiviruses.

Two different sites known to be critical for primate lentivirus budding have been identified in SIV Gag p6 protein sequences - PT/SAP and YPXL. All *Cercopithecus* SIVs have both motifs, except SIVdeb and SIVden [27, 87] which, like SIVcol, only have a YPXL motif. On the other hand, SIVcpz, SIVlho, SIVsun, SIVmnd-1, SIVsmm, SIVagm, SIVrcm and HIV-2 encode only for a N-terminal PT/SAP Tsg101 binding motif. The presence of one motif compensates for the lack of the other, but to date the functional significance of the occurrence of both motifs remains unknown [229, 304]. The alignment of SIVwrc*Pbt*-05GM-X02 with other SIV Gag p6 protein sequences revealed the presence of both PT/SAP and YPD/SL motifs (Table 7.2).

We then inspected the five variable regions designated V1 to V5 and the CD4 binding domain of the gp120 envelope glycoprotein. As in other primate lentiviruses, the V1 region of the Env protein of SIVwrc*Pbt*-05GM-X02 revealed high levels of variability, insertion/deletion polymorphism, and the characteristic presence of threonine residues. The CD4 binding domain was conserved and 3 amino acid insertions were observed, which is similar to SIVdrl, SIVmnd-1, SIVlho, SIVsun, SIVmnd-2 and SIVcol (data not shown).

All primate lentiviruses, including HIV, have at least eighteen conserved cysteine residues in the extracellular envelope domain. They form paired disulfide bonds responsible for the conformational structure of the virus surface and for the envelope functions, including the interaction with the CD4 receptor on the host cell surface [27]. All SIVs derived from *Cercopithecus* species (SIVdeb/SIVsyk/SIVgsn/SIVmon/SIVmus), SIVcpz and SIVcol contain 18 conserved cysteine residues. SIVs belonging to the other lineages (SIVagm/SIVlho/SIVsmm/SIVrcm/SIVmnd/SIVdrl) encode for additional pairs of

cysteine residues. SIVwrc*Pbt*-05GM-X02 encodes for three additional cysteine pairs, which fell within variable domain 2 and between variable domains 3 and 4 at similar positions as the additional cysteines observed in the SIVlho, SIVsun, SIVmnd-2 and SIVdrl lineages.

Table 7.2: Gag P6 motifs after alignment of primate lentiviral Gag p6 protein sequences

| SIV strain | Gag P6 motifs | |
|---|---|---|
| SIVtalCM8023 | PTAP | YPSL |
| SIVtalCM266 | PSAP | YPSL |
| SIVdebCM5 | - | YPDL |
| SIVdebCM40 | - | YPDL |
| SIVgsnCM166 | PTAP | YPSL |
| SIVgsnCM71 | PSAP | YPSL |
| SIVmonCML1 | PSAP | YPSL |
| SIVmusCM1085 | PTAP | YPSL |
| SIVden | - | YPSL |
| SIVmusCM1239 | PTAP | YPSL |
| SIVrcmNgm | PTAP | - |
| SIVrcmGAB1 | PSAP | - |
| SIVdrl1FAO | PSAP | YNSL |
| SIVmnd14cg | PSAP | YNSL |
| SIVsykKE51 | PTAP | YPSL |
| SIVsyk173 | PSAP | YPSL |
| SIVcpzTAN1 | PTAP | - |
| SIVlho | PSAP | - |
| SIVsunL14 | PSAP | - |
| SIVmndGB1 | PTAP | - |
| SIVsmm251 | PTAP | - |
| SIVagmGRI677 | PTAP | - |
| SIVagmTAN1 | PTAP | - |
| SIVagmVER155 | PSAP | - |
| SIVcpzUS | PTAP | - |
| HIV2BD205 | PSAP | - |
| SIVcolCGU1 | - | YPSL |
| SIVwrc-05GM-X02 | PTAP | YPSL |

## 7.5 Discussion

In this study we showed that geographically isolated subspecies of the western red colobus (*Piliocolobus badius*) in The Gambia and Côte d'Ivoire are both infected with closely related species-specific SIVs, designated SIVwrc. We obtained the full-length genome sequence of a SIVwrc strain from an individual of Temminck's red colobus (*Piliocolobus badius temminckii*) in The Gambia and showed that SIVwrc is a species-specific lineage, although distantly related to the SIVlho and SIVsun

lineages across its entire genome. Interestingly, in *gag* and in the 5'end of pol, SIVwrc was also distantly related to SIVcol isolated from a mantled guereza (*Colobus guereza*) from Cameroon, the only other fully characterized SIV in the subfamily Colobinae.

The habitat of *P.b.temminckii* spans the regions of Senegal, The Gambia, Guinea Bissau and NW Guinea whereas the *P.b.badius* range is limited by the mountainous areas of Sierra Leone, the Niger river in north-east Guinea and the Sassandra river in Côte d'Ivoire [187]. However, the coastal forests of Guinea have not been extensively surveyed; therefore we cannot exclude the possibility that a red colobus population exists that connect these two subspecies. Pelage and vocalization data indicate a close relationship between *P.b.badius* and *P.b.temminckii*. Mitochondrial lineages are paraphyletic with respect to one another [374]. This reflects the results we obtained from the partial *pol* and *env* SIVwrc fragments and suggests that these two subspecies may either still be in contact or only recently diverged. A study conducted on red colobus monkeys in Taï National Park, Côte d'Ivoire, shows that they represent a substantial reservoir of SIVwrc*Pbb*, with at least 25% of the adult population infected [220] . None of the 16 faecal samples from Temminck's red colobus analysed in this study by RT-PCR resulted to be positive. However, the limited amount of material available allowing only a single RNA extraction for each sample, together with several thaw and freeze procedures and the low sensitivity of viral RNA detection previously reported, do not allow us to provide any significant information of infection rates in the Abuko population of Temminck's red colobus [217, 220].

Phylogenetic analysis of the full-length genome showed that SIVwrc forms a distinct lineage and it is most closely related to the SIVlho/sun/mnd-1 lineage across the entire genome, although to a lower extent in the 3' end of *pol*. Interestingly, SIVcol clusters also with SIVwrc and the above mentioned SIVs in *gag* and especially in the 5'end of *pol*. Since their hosts (subfamily Colobinae) are phylogenetically related, the relationship between these SIV lineages may reflect a common ancestry of their genomes.

Overall, the phylogenetic relationships between the different SIVs and the presence of specific functional motifs, with the exception of the motifs in *gag* typical of the *Cercopithecus* lineage, suggest that SIVwrc has an ancestral link with the SIVs

from l'Hoest and sun-tailed monkeys. This relationship is surprising because of the geographical separation of the different hosts of these SIV lineages. However, the African continent has been subjected to periodic episodes of climatic change that mediated alternating phases of tropical rainforest contraction and expansion throughout the Plio-Pleistocene (from about 2.9 to 0.8 Ma) [89], thereby creating montane and lowland forest remnants or 'refuges.' These events have certainly affected the speciation and current geographic distribution of primates, and they have possibly also influenced the spreading patterns of an SIV ancestor. The territories we inspect today may have been inhabited by different or more primates species in the distant past. For example, the olive colobus is a relict species confined to the forests of West Africa, whereas the widely distributed fragmented populations of red colobus show that these animals once ranged across the forested parts of Africa; the black-and-white colobus (genus *Colobus*) is also widely distributed across equatorial Africa.

In order to better understand the evolution of SIVs in the colobines, it will be important to characterize additional SIVs in the olive colobus (*Procolobus verus)* and the remaining species of *Colobus* and *Piliocolobus* across Africa. Particular attention should be paid to *Piliocolobus* species whose ranges overlap today with those of the *Cercopithecus* species harbouring SIVlho and SIVsun. This will help to determine whether the virus emerged before or after red colobus speciation events and will provide further insight into the importance of biogeographic barriers and cross-species transmission in SIV evolution. Geographic barriers can influence evolution of SIVs among different species, but also within a single species, as is shown for mandrills (*Mandrillus sphinx*) in Gabon. Based on partial *pol* and *env* sequences, the two geographically separated western red colobus subspecies seem to be infected with a similar SIVwrc variant. However, full-length characterization of SIVwrc*Pbb* is necessary to confirm this observation. Geographic isolation, human presence and ecological factors like vegetation type and distribution can shape or elicit new or different behaviours, which can play a role in cross-species transmission and recombination of divergent SIVs. For example, contrary to most colobus species which are mainly arboreal, Temminck's red colobus has been observed to spend relatively long periods of time at ground level [117] partly because populations in these regions do not face human or chimpanzee predation pressure, which is in

contrast to the red colobus population in Taï Forest in Côte d'Ivoire [31, 33, 310], but also because Temminck's red colobus has adapted to an increasingly dry and fragmented ecosystem [118]. One of the consequences of this diminished predation is that Temminck's red colobus have been observed in polyspecific association with ground living primates like patas monkeys (*Erythrocebus patas*) and green monkeys (*Cercopithecus aethiops sabaeus*) [117, 354].These polyspecific associations are thus different than those observed for the *P.b.badius* subspecies in the Tai forest.

Overall, our results confirm the complex evolutionary history of primate lentiviruses, which has been driven by host-virus co-speciation, cross-species transmission and recombination events over an extended period of time. Characterization of additional SIVwrc viruses is needed to understand the ancestral phylogenetic relation to SIVs from l'Hoest and sun-tailed monkeys and whether recombination occurred between ancestors of the SIVwrc and SIVlho/sun lineages. In addition, western red colobus are heavily hunted by the human population and given the relatively high frequency of SIVwrc infection in the wild, we should keep this virus under investigation for potential cross-species transmissions.

## 7.6 Acknowledgements

We thank the Department of Parks and Wildlife, The Gambia. Funding for this project was provided by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de Recherches pour le SIDA (ANRS).

# 8. Molecular characterization of a novel simian immunodeficiency virus lineage (SIVolc) from olive colobus (*Procolobus verus*) and of two new SIVwrc strains from western red colobus monkeys (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire

Florian Liegeois[1], Bénédicte Lafay[2], Sabrina Locatelli[1], Pierre Formenty[3], Eric Delaporte[1] and Martine Peeters[1]

1-UMR 145, Institut de Recherche pour le Developpement (IRD), and University of Montpellier 1, Montpellier, France[1]

2-UMR CNRS-IRD 2724, Montpellier, France[3]

3-Ebola Taï Forest Project, Abidjan CI, World Health Organization (WHO), Department of Communicable Diseases Surveillance and Response(CDS/CSR)

**This is an article in preparation**

## 8.1 Abstract

Simian immunodeficiency viruses (SIVs) are found in an extensive number of African primates and humans continue to be exposed to these viruses by hunting and handling of primate bushmeat. Here, we report the full length molecular characterization of a new SIV lineage, SIVolc, from a wild-caught olive colobus (*Procolobus verus)* as well as two new strains from western red colobus (*Piliocolobus badius badius*) from the Taï Forest, in Côte d'Ivoire. Phylogenetic analyses of 2 full-length genome sequences revealed that the SIVwrc*Pbb* strains were closely related to the newly described SIVwrc*Pbt* from The Gambia and confirmed that SIVwrc represents a new distinct SIV lineage. Phylogenetic analyses of the SIVolc full-length genome revealed that this virus belongs to a distinct SIV lineage, but it is closely related to SIVwrc across its entire genome.

## 8.2 Introduction

With the exception of SIVcpz from central and eastern chimpanzees and the recently discovered SIVgor from West-central gorillas, all non-human primate lentiviruses have been isolated from African Old World monkeys (Cercopithecidae). Cercopithecidae are subdivided into two distinct subfamilies, the Colobinae and the Cercopithecinae [139]. Colobinae are subdivided into an African and an Asian group. African colobinae are represented by three genera; *Colobus*, *Piliocolobus* and *Procolobus* [139]. Colobinae separated from the other Old World monkeys at least 16 million years ago [305]. Their habitats ranges all over the equatorial forested parts of Africa, except for the olive colobus (*Procolobus verus*), which is confined to the tropical forest in West Africa [187].

SIVs have been isolated from all African colobinae genera. SIVcol, from black and white colobus (*Colobus guereza*) in Cameroon and SIVwrc*Pbt*, from western red colobus (*Piliocolobus badius temminckii*) in The Gambia have been fully molecularly characterized [78, 218]. SIVcol represents the most divergent SIV so far identified [78]. Partial SIVwrc*Pbb pol* and *env* sequences isolated from western red colobus (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire, have been previously described [77]. Phylogenetic analyses of these SIVwrc*Pbb* fragments with the recently described SIVwrc*Pbt* showed that both SIVwrc isolated from geographically

separate *Piliocolobus badius* subspecies formed a species specific monophyletic cluster named SIVwrc lineage [218].Interestingly, SIVwrc*Pbt* was also closely related to viruses from the l'Hoest lineage. Conversely, phylogenetic analyses of SIVolc partial *pol* sequences showed that it could potentially represent a distinct species-specific SIV lineage [77].

To further document the evolutionary history and phylogenetic relationship between SIVs from colobinae genera and other SIV representatives, we have characterised here two full-length genomes of SIVwrc and one of SIVolc from western red colobus (*Piliocolobus badius badius*) and olive colobus monkeys (*Procolobus verus*) living in the Taï Forest in Côte d'Ivoire. These primate species, and in particular the western red colobus monkeys, are heavily hunted by chimpanzees and by the human population. The SIVs they harbour could have the potential to cross the species barrier.

## 8.3 Materials and Methods

### Primate specimens and serologic testing

Whole blood was obtained from animals anesthetized using tele-injection rifles (telinject GUT 50) and tissues from carcasses found on forest floor of the Taï Forest by sanitary surveillance patrols or by primatologists. These samples were collected between 1997 and 2000, following an Ebola outbreak affecting chimpanzee populations in the region [77, 108]. Samples were first stored in liquid nitrogen and later kept at -70℃. The identification of the monk eys was done in the field and confirmed by analysis of the skulls. Whole-blood and tissue samples from two western red colobus (*98CI-04, 97CI-14*) and one olive colobus (*97CI-12*) were available for this study. Serological INNOLIA-HIV confirmation tests and partial *pol* PCRs identified these samples as SIV positive [77].

### PCR amplification and sequencing of SIVwrc and SIVolc full-length genomes

DNA was extracted from whole blood and lymph nodes using the Qiamp®blood and Qiamp®tissue kit. RNA was extracted from plasma for sample 98-CI04 using the QIAamp®Viral RNA Mini Kit according to the manufacter's instructions (Qiagen S.A, Courtaboeuf, FR). Complete SIVwrc98CI-04*,* SIVwrc97CI-

14 and SIVolc97CI-12 genomes were obtained by amplification of overlapping PCR fragments and unintegrated circular DNA using combinations of specific SIVwrc and SIVolc primers as well as degenerate SIV primers. The corresponding amplification strategies are depicted in figures 8.1a and 8.1b. RT-PCR and PCR amplifications were done using the Expand Reverse Transcriptase and the Long Expand PCR kit respectively (Roche Applied Science, Indianapolis, IN) according to the manufacter's instructions. Each amplification reaction included a manual hot-start and 35 to 40 cycles. Annealing temperatures were set according to the primer melting temperatures and extension times varied depending on the size of the expected fragment and were typically set at 1mn/kb. PCR products were agarose gel purified and directly sequenced using cycle sequencing and dye terminator methodologies [ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq FS DNA polymerase (PE Biosystems, Warrington, England) on an automated sequencer (ABI 3130*XL*, Applied Biosystems)] using a primer walking approach. To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences by using SEQMAN DNASTAR software (lasergene, DNASTAR, Inc., Madison, WI).

**Similarity plots and phylogenetic analyses**

Nucleotide and protein sequences were aligned using MEGA3 and ClustalX 1.8 [198, 372], with minor manual adjustments. Proteome sequences were generated by joining deduced Gag, Pol, Env and Nef amino acid sequences. The predicted protein sequences encoded by SIVwrc*Pbb* and SIVolc were compared to representative of known HIV/SIV lineages. We performed similarity plot analysis to determine whether the newly characterised SIVwrc and SIVolc sequences were recombinant with any of the other SIV lineages (SIMPLOT package version 2.5 [296]). We used a sliding window of 200 amino acids (aa) moved in steps of 20 aa. Phylogenetic tree analyses were done on amino acid sequences and regions analysed were defined on the basis of simplot results observed (Gag (390 aa), Pol1 (279aa), Pol2 (286 aa), Pol3 (355 aa) and Env (560aa)). Phylogenies were inferred by the Bayesian method implemented in Mr Bayes v3.1 [419] using the rtRET model of protein evolution [90] with gamma distributed rates at sites [421]. The program was run for 3, 5 and 6 million generations for Gag, Pol (Pol 1, Pol 2 and Pol 3) and Env genes respectively, including a "burn in" of 10 percent. Bayesian likelihoods and

parameters were examined with the Tracer program (http://evolve.zoo.ox;ac.uk/software.html).

**RNA Secondary structure predictions**

The TAR RNA secondary structure was predicted and drawn using the GENQUEST DNASTAR package (Lasergene, DNASTAR, inc, Madison, WI).

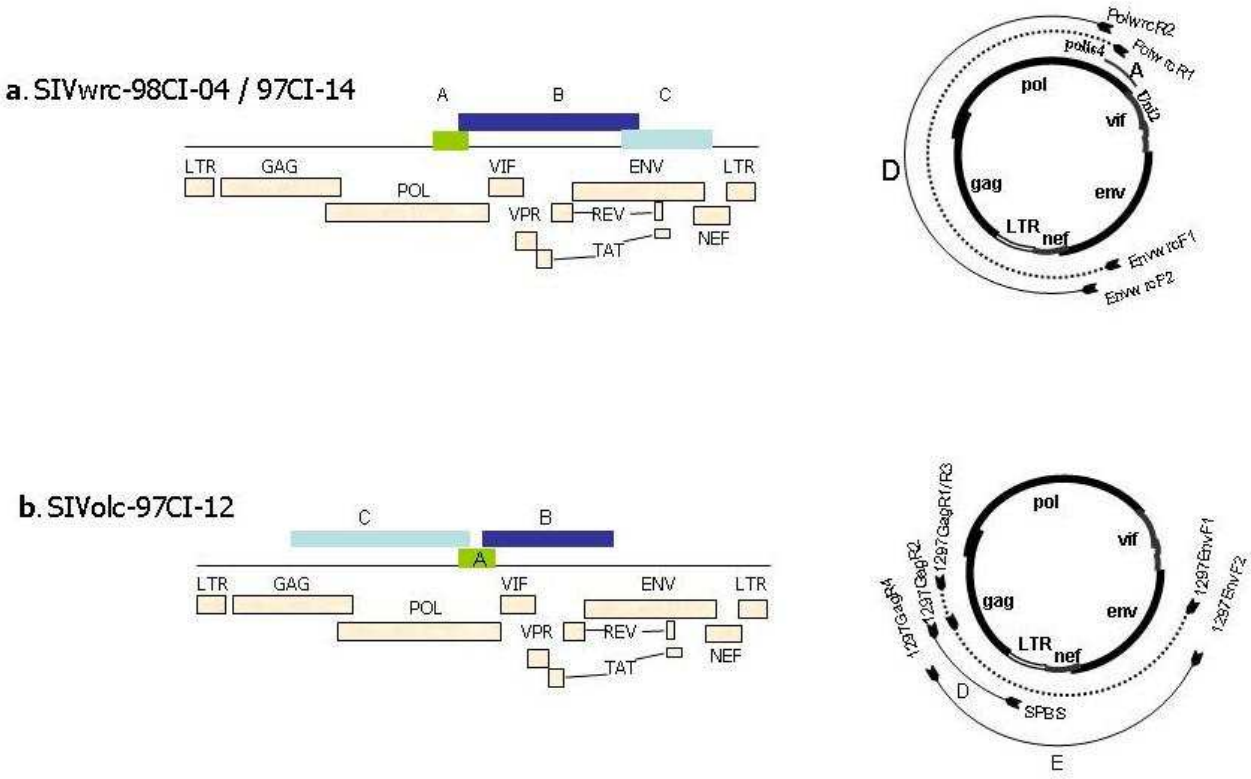



Figure 8.1: PRC amplification strategies for overlapping fragments shown to the left and for unintegrated circular DNA shown to the right of the figure

## 8.4 Results

### Similarity plots

Figure 8.2a depicts similarities between SIVwrc98CI-04 and SIVwrc97CI-14 as well as between one representative of the SIVwrc*Pbb* (SIVwrc98CI-04 ) against other SIV lineages. SIVwrc*Pbb* were closely related to the newly characterised SIVwrc*Pbt* from The Gambia across their entire genomes [218]. As described for SIVwrc*Pbt*, SIVwrc*Pbb* strains were more closely related to the SIVlho lineage than to any another SIV lineage, in particularly in two parts of their genomes: in the 5'part of the Pol gene and across the entire Env gene.

Figure 8.2b shows similarities between SIVolc from olive colobus and the other representative SIV strains. SIVolc is clearly related to SIVwrc along Pol and Env genes and, as observed for SIVwrc strains, to the SIVlho lineage in the 5'part of the Pol gene and in the entire Env gene.

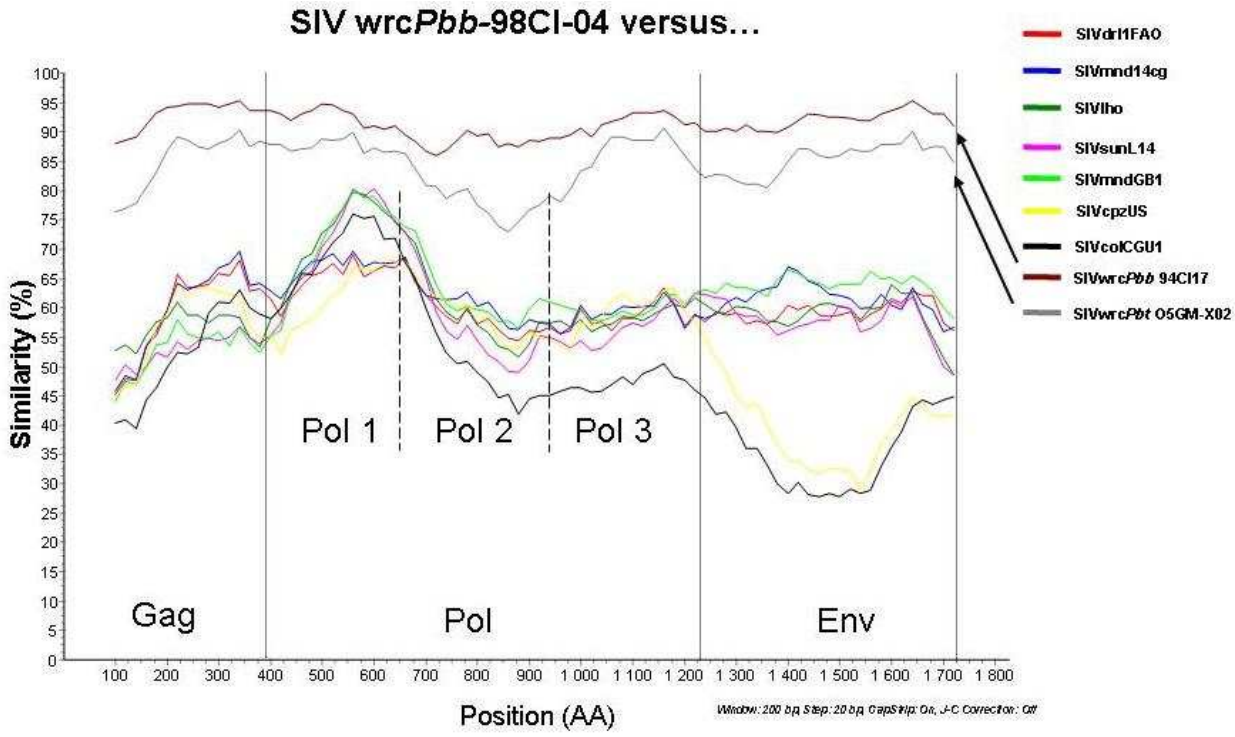



Fig. 8.2a

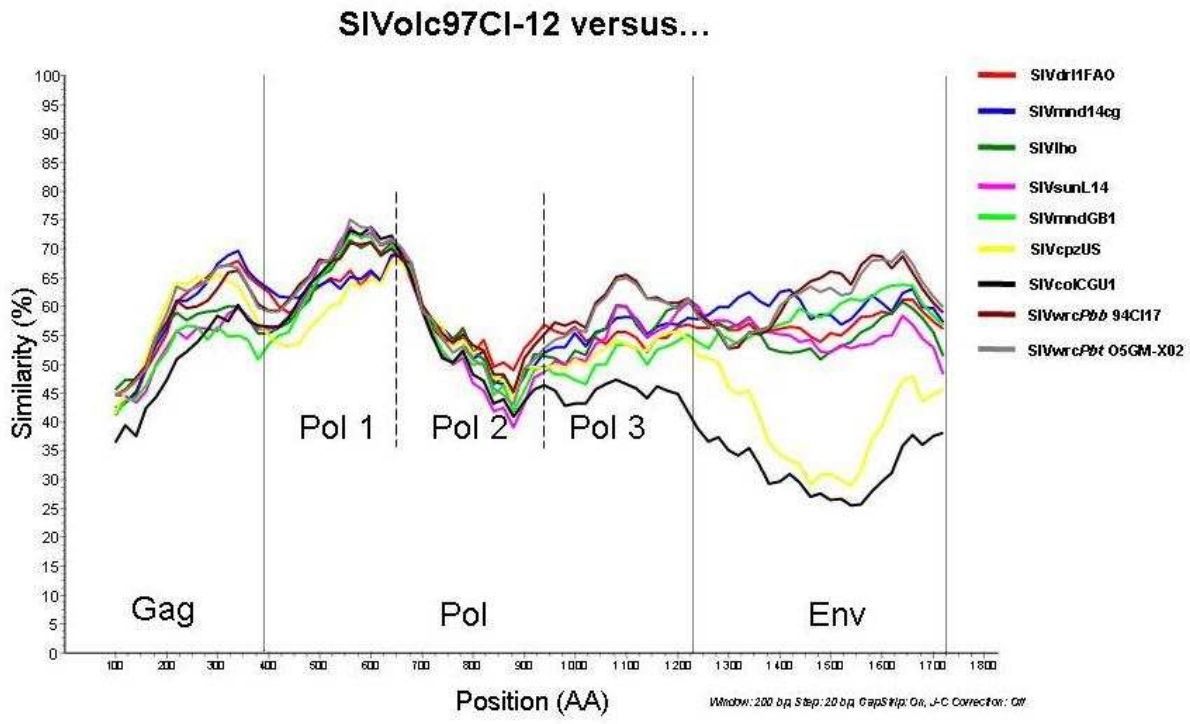



Fig. 8.2b

Figure 8.2: Similarity plots of concatenated Gag, Pol1, Pol2, Pol3, and Env protein sequences. It shows the extent of genetic similarity between SIVwrc*Pbb*-98CI-04 (a) and SIVolc97CI-12 (b) versus other SIV lineages. The proportion of amino acid sequence similarity per 200 residues window (vertical axis) is plotted against the midpoint of the sequence window (horizontal axis).

**Phylogenetic analyses of full-length SIVwrcPbb and SIVolc genomes**

Phylogenetic trees show that SIVwrc*Pbb* and SIVolc are distinct SIV lineages, but closely related across their genomes. SIVwrc*Pbb* forms a monophyletic clade in each of the three major genes (Fig 3 a, b, c, d, e) as well as in accessory genes (data not shown) together with the recently described SIVwrc*Pbt* [218]. For each gene analysed, both SIVwrc and SIVolc lineages clustered together with the SIVlho lineage, although with a lower posterior probability in Gag. SIVolc conserves a basal position within the SIVwrc/olc clade in every phylogenetic tree, except in Pol2. SIVcol from *Colobus guereza* clusters together with SIVwrc, SIVolc and SIVlho lineages in Gag and Pol 1, although with a higher degree of divergence in Gag (Figs. 4a, 4b). Finally, in Env SIVwrc and SIVolc form a highly supported cluster with the SIVlho lineage and also with SIVmnd-2 and SIVdrl.

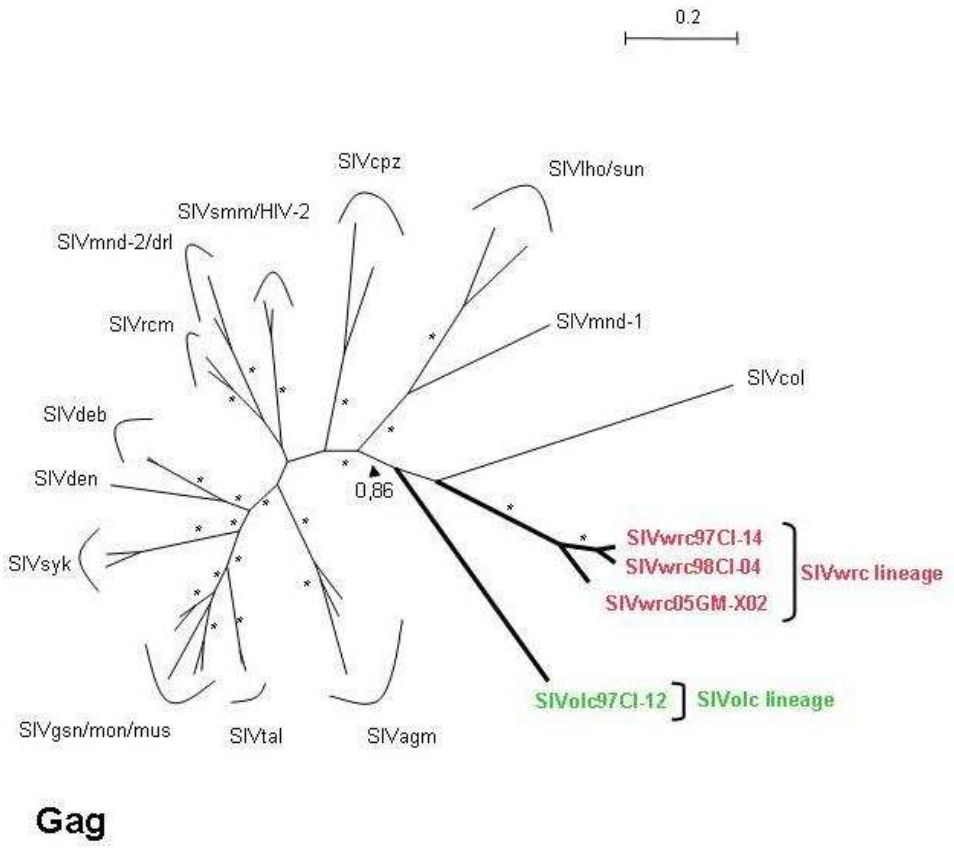



Figure 8.3a

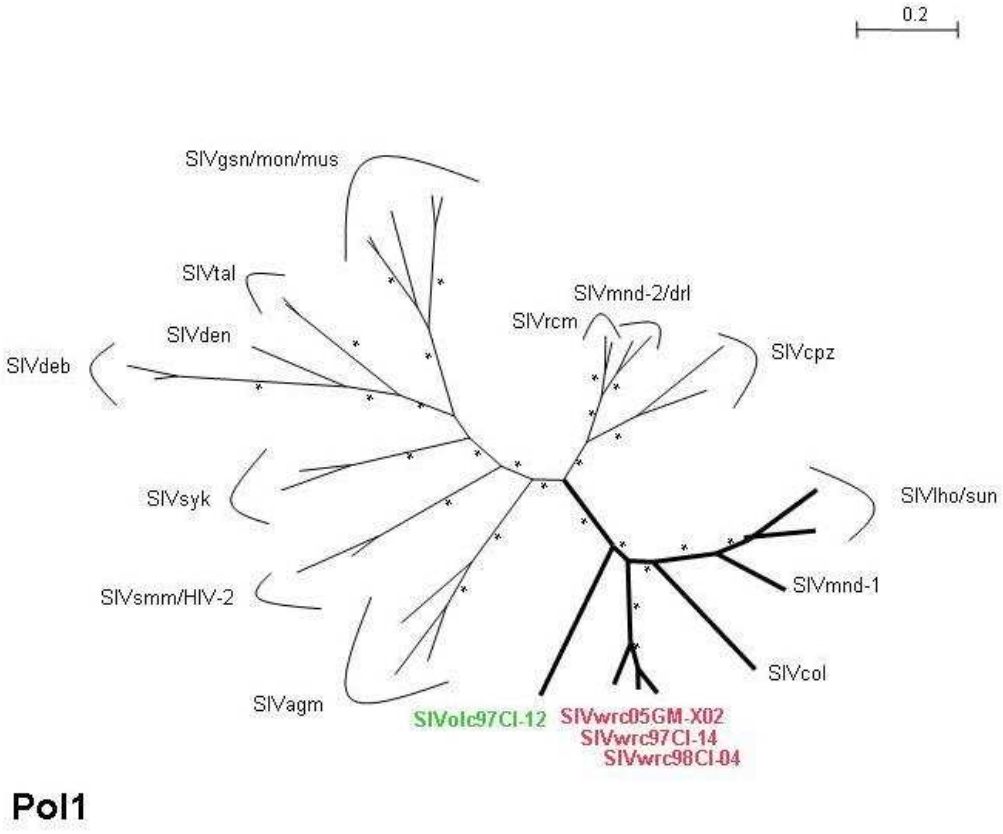



Figure 8.3b

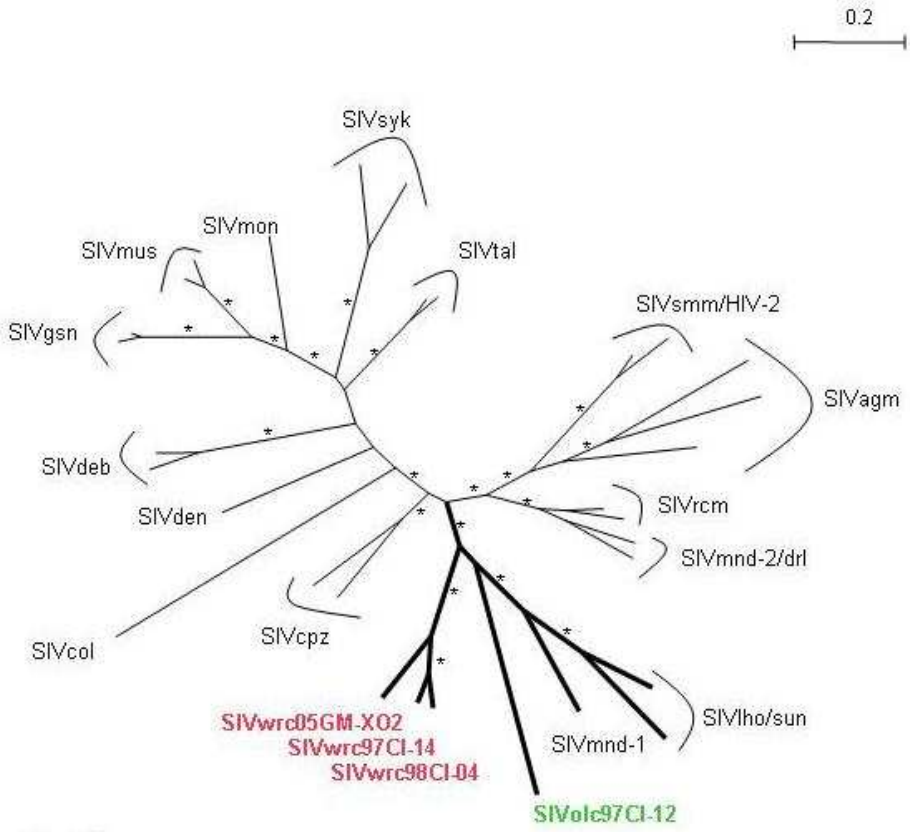



Figure 8.3c

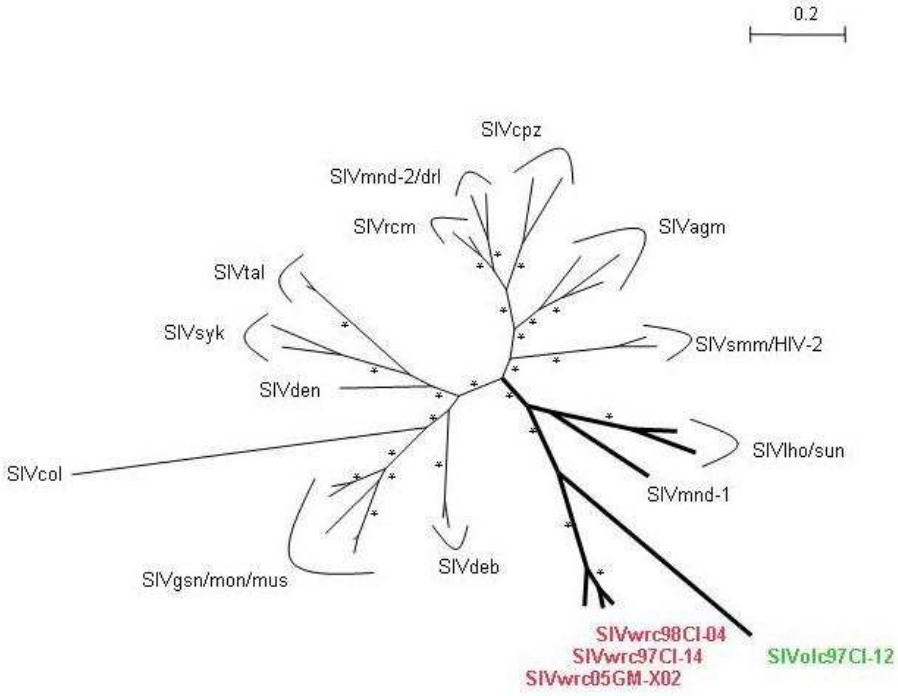
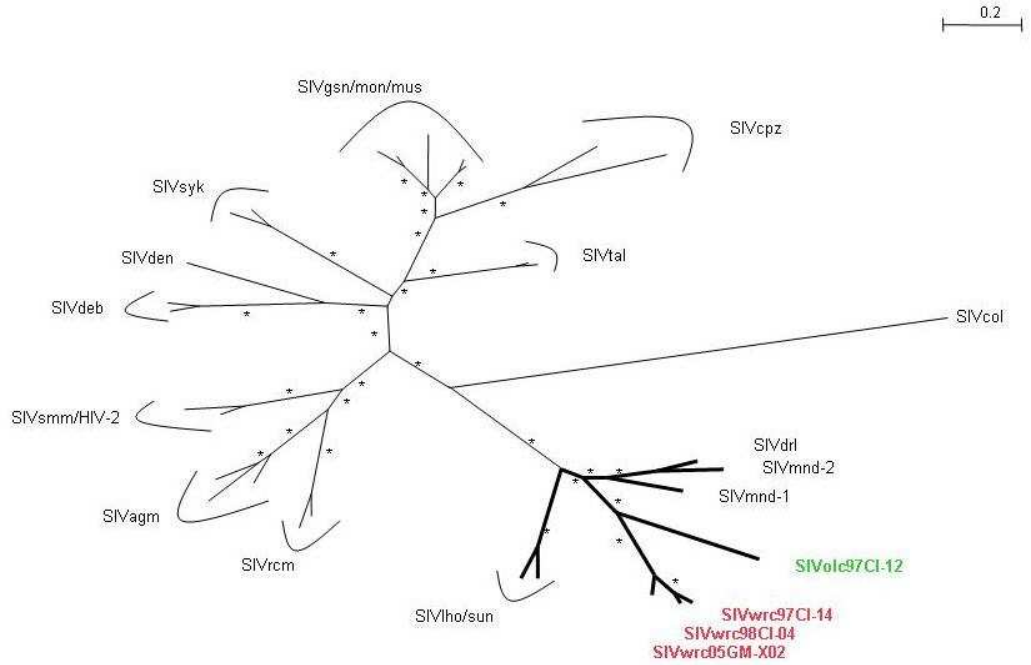



Figure 8.3d




## Env

Figure 8.3e

Figure 8.3 (3a, 3b, 3c, 3d, 3e): Phylogenetic relation of SIVolc and SIVwrc to other SIV lineages in Gag, Pol1, Pol2 and Env. Stars at nodes represent posterior probabilities estimated by the Bayesian method. Only those ≥ 92% are shown. Scale bars indicate number of substitution per site.

### Genomic organisation and functional motifs of SIVwrcPbb and SIVolc lineage

We compared SIVwrc*Pbb* and SIVolc genomes with other primate lentiviruses and they showed the expected reading frames for *gag, pol, vif, vpr, tat, rev, env* and *nef*. SIVwrc and SIVolc did not encode for a *vpu* or *vpx* analogue.

The SIVwrc*Pbb* and SIVolc long terminal repeats (LTR) contain all the characteristic features of other primate lentivirus LTRs including TATA, NF-kB site and potential SP-1 regions (data not shown).

The secondary structure prediction of SIVwrc*Pbb* tar element showed an unusual organisation with a double stem-loop structure consisting of a 3 nucleotides (GCC) and a single nucleotide bulges (U), a 7-bp and a 6-bp stems having a 5-bp identical terminal loop with the sequence 5'-CUGGU-3' (data not shown). This tar element is quite similar to the tar element described for the SIVwrc*Pbt*, which reinforces the common origin of these viruses. SIVolc has a typical predicted

secondary structure of tar element with two stem-loops consisting of 2 identical nucleotide bulges (UU) and two 6-bp stems having a 6-bp terminal loop with the sequences 5'-CUGAGU-3' and 5'-CUGGGU-3' respectively (data not shown).

SIVwrc*Pbb* and SIVolc contain in the gp120 envelope subunit the same conserved 18 cysteine residues found in all other primate lentiviruses and display, at similar positions, the same additional cysteine residues found in SIVlho lineage, SIVdrl/mnd-2 and SIVwrc*Pbt*.

Finally, similarly to all other primate lentiviruses, except SIVcol, SIVden and SIVdeb, SIVwrc*Pbb* and SIVolc viruses have a PT/SAP Tsg101 binding motif site. In the absence of this motif, budding occurs *via* binding of the YPXL motif with high affinity to AIP1, a second host protein involved in endosomal sorting and retroviral budding [229, 304]. Both motifs (PT/SAP and YPXL) are found in *Cercopithecus* and *Miopithecus* SIV lineages with the exception of SIVdeb and SIVden [27, 87, 215]. They were proposed to represent a specific signature for the SIV-*Cercopithecus* / *Miopithecus* lineage [27], untill both motifs were also described in the recently published SIVwrc*Pbt* [218]. In addition, also SIVolc has both YPXL and PT/SAP motifs, weakening the SIV-*Cercopithecus* hypothesis. In SIVwrc*Pbb* Gag p6 protein sequences the YPXL motif was absent, but was replaced by a WPXL motif.

## 8.5 Discussion

We have characterised in this study the full-length genome of two strains of SIVwrc isolated from the *Piliocolobus badius badius* subspecies found in Côte d'Ivoire and we confirm here their close phylogenetic relationship with the SIVwrc strain isolated in the subspecies *Piliocolobus badius temminckii* from The Gambia. These subspecies are geographically separated, although their ranges are poorly documented and could possibly overlap around the coastal forest of Guinea [140]. Mitochondrial lineages showed that *P.b.badius* and *P.b.temminckii* form a paraphyletic clade within the red colobus species [374]. Although a virus-host co-evolution seems to be the most plausible hypothesis for the emergence of SIVwrc in these two subspecies, we cannot exclude yet that SIVwrc could have been acquired by cross-species transmission, especially given the close phylogenetic relationship with SIVs from the l'Hoest lineage.

The SIVolc strain isolated from olive colobus monkeys is more closely related to SIVwrc than to other SIV lineages across the whole genome, but displays nonetheless a great genetic diversity and it is therefore distinct from SIVwrc. We need to characterize additional SIVolc strains form different geographic areas, in order to confirm its phylogenetic position in respect to SIVwrc: whether these two species have been infected by a common SIV ancestor and virus-host co-speciation has followed, or there has been a more recent transmission from one species toward the other. Olive and red colobus monkeys are sympatric in the Taï Forest, but no feeding competition has been observed [192] and, in general, they use different strata of the forest canopy. Olive colobus are notorious for associating constantly with a Diana monkey group as an anti-predation strategy, therefore a viral exchange between these two species is possible. To date, only serological evidence of SIV has been described for Diana monkeys [223]. The basal position of SIVolc within the SIVwrc/olc tree branch suggests an earlier divergence compared to SIVwrc: this is more in favour of a virus-host co-speciation.

These speculations notwithstanding, SIVwrc and SIVolc are both closely related to the SIVlho lineage across the whole genome. The two *Piliocolobus* subspecies and olive colobus are geographically separated from *Mandrillus sphinx*, *Cercopithecus lhoesti* and *Cercopithecus solatus* infected with SIVmnd-1, SIVlho and SIVsun respectively (all three belonging to the SIVlho lineage). Within the species infected with SIVs from the l'Hoest lineage, *C.lhoesti* is also geographically separated from *M.sphinx* and *C.solatus*. To date, the relationship between SIVlho/sun and SIVmnd-1 remains an enigma and cannot be explained by host-dependent evolution (mandrills and l'Hoest monkeys are phylogenetically distant species within the *Cercopithecinae*) as well as by recent cross-species transmission because: (i) mandrills and l'Hoest monkeys inhabit geographically separate regions of Central Africa, (ii) SIVsun from *C. lhoesti solatus* monkey, whose ranges overlap with that of the mandrill, is more distantly related to SIVmnd and could not have been the proximal source of SIVmnd. Different hypotheses tried to explain this close relationship: one focuses on a yet unidentified species of *Piliocolobus* which could establish the SIV phylogenetic link. *Piliocolobus* species range discontinuously from the West African forests through Central-eastern Africa. In Cameroon, *Piliocolobus penantii preussi* shares the same habitat with mandrills and *C. lhoesti preussi*

monkeys and *Piliocolobus* species' range overlaps that of *C. lhoesti lhoesti* monkey in the eastern forests of the Democratic Republic of Congo.

In the Env phylogenetic tree, SIVwrc was more closely related to SIV-mnd-1, SIVmnd-2/drl than to SIVlho/sun. This suggests a cross-species transmission between SIVwrc and SIVlho/SIVsun or between their respective ancestors. It remains a matter of speculation whether SIVwrc, SIVlho/SIVsun or both lineages are recombinants.

By characterising further SIVs from other species of *Colobus* and *Piliocolobus* monkeys we could better understand if the close relationship between SIVwrc, SIVolc, SIVlho and SIVcol in the first part of *pol* gene is the result of an ancestral SIV infecting the Colobus genus and consequently better understand the origin of the SIVlho lineage.

We also need to isolate and characterise other SIVs in red colobus not only from the western assemblage, but also from the Central and eastern ones to determine whether SIVwrc is a virus confined to West Africa or if a common SIV ancestor coevolved and spread among all species of red colobus across Africa.

In order better to understand the phylogenetic relationship between SIVwrc, SIVolc and SIVs from the l'Hoest lineage, we should isolate and characterize SIVs potentially infecting species inhabiting the forests of West-central Africa: *Pilocolobus pennanti* subspecies and also other *Cercopithecus lhoesti* subspecies such as *C. lhoesti preussi* from Cameroon and *C. lhoesti insularis* from Bioko Island.

Finally, the presence of both PT/SAP and YPDL motifs has been proposed as a specific SIV-*Cercopithecus* signature. We report here SIVs from the Colobinae genus, which have both motifs. Therefore the presence of both motifs cannot be considered anymore as a specific SIV-*Cercopithecus* signature. With the isolation and characterisation of new SIVs, it becomes more and more difficult to clearly disentangle the phylogeny of primate lentiviruses from that of their hosts. This can only confirm that the evolutionary history of primate lentiviruses has been driven by host-virus co-speciation, cross-species transmission and recombination events over an extended period of time.

## 8.6 Acknowledgements

We thank the ‘Ministère d’Enseignement Supérieur et Recherche Scientifique’, the ‘Ministère d’Agriculture et Ressources Animales’, the ‘Centre Suisse de Recherche Scientifiques’(CSRS) in Abidjan, and the ‘P.A.C.P.N.T.’ and the ‘Centre de Recherche en Ecologie’ in Côte d’Ivoire for permission to conduct research in the Taï National Park.

This study was financially supported by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de Recherches pour le SIDA (ANRS).

# 9. Lack of evidence of simian immunodeficiency virus infection among several wild-living primate species in Taï National Park, Côte d'Ivoire: significance of demographic, ecological and behavioural data and limitations of non-invasive research

Sabrina Locatelli[1], Amy D. Roeder[2], Michael W. Bruford[2], Ronald Noë[3], Eric Delaporte[1], Martine Peeters[1]

[1]UMR 145, Institut de Recherche pour le Développement (IRD), and University of Montpellier 1, Montpellier, France

[2]Cardiff School of Biosciences, Museum Avenue, Cardiff CF10 3TL, UK

[3]Ethologie des Primates (DEPE-IPHC-CNRS UMR 7178) and University of Louis Pasteur, Strasbourg, France

**This is an article in preparation**

## 9.1 Abstract

The Taï Forest, in Côte d'Ivoire, is home to sooty mangabeys (*Cercocebus atys*) and western red colobus (*Piliocolobus badius badius*), which are infected with simian immunodeficiency virus (SIV) at a high rate. Taï SIVsmm strains from sooty mangabeys cluster with five of the eight recognized groups of HIV-2 and western red colobus carrying SIVwrc are heavily hunted by the chimpanzees as well as by the human population, a risk factor for SIV cross-species transmission. The Taï Forest is inhabited by 7 additional diurnal non-human primates species. We collected a total of 127 faecal samples from two colobus species (*Colobus polykomos* and *Procolobus verus*) and four *Cercopithecus* species (*C. diana, C. campbelli, C.petaurista* and *C. nictitans*). We tested these samples for HIV cross-reactive antibodies using the INNO-LIA HIV I/II score confirmation assay and we also performed Reverse Trancriptase-Polymerase Chain Reactions (RT-PCR) targeting the *gag, pol* and *env* regions of the SIV genome. All faecal samples tested with the INNO-LIA assay resulted negative or non interpretable. We discuss here the limitation of faecal sample analysis as well as risk factors of SIV infection in non-human primate populations, considering previously acquired behavioural ecology data. The species vulnerability to human hunting pressure and the potential for cross-species transmission are also addressed.

**Keywords:** Taï National Park, faecal analysis, SIV, non-human primates, serologic assays, primates social structure, non-invasive sampling

## 9.2 Introduction

There are at least 40 different non-human primate (NHP) species in sub-Saharan Africa which are infected with SIVs [382]. It is widely accepted today that SIV infecting chimpanzees (*Pan troglodytes troglodytes*) and western gorillas (*Gorilla gorilla gorilla*) in West-central Africa represent the reservoir of simian immunodeficiency viruses (SIVcpz*Ptt* and SIVgor) [122, 386], which have crossed at least three times the species barrier to humans, resulting in the emergence of HIV-1 group M responsible for the AIDS pandemic affecting more than 60 million people, HIV-1 group N, infecting only a few individuals in Cameroon [13] and HIV-1 group O - like viruses, which infections remain mostly confined in Cameroon and neighbouring countries [12, 289]. The progenitor of human immunodeficiency virus type 2 (HIV-2), an epidemic mostly confined to West Africa, has been recognised in the simian immunodeficiency virus of sooty mangabeys (*Cercocebus atys atys*) (SIVsmm), which has been transmitted to humans at least on 8 occasions [63, 143, 322].

African primates are natural hosts for these viruses and the proportion of animals that are seropositive in the wild can be quite high [2, 183, 220, 223, 275, 322, 385]. In general, infected primates do not seem to develop any clinical symptoms [113, 312]. The reasons for this lack of pathogenicity are still not well understood. However, it has become widely accepted that the handling and the consumption of SIV infected primates participated to the emergence of HIV and that human beings are still exposed to SIVs when adopting these practices [287]. Therefore, to better understand the origins of HIV-1 and -2 and to assess the potential for additional lentiviruses to infect the human population, it is important to continue screening new SIV strains.

Since new methods to detect specific antibodies and nucleic acids from faecal matter have been developed, researchers have started to investigate the presence of SIV infection in wild-living populations by analysing samples collected from the forest floor. This non-invasive method has been adopted to investigate SIV prevalence mainly in ground - dwelling wild-living NHPs [183, 322, 385, 386]. Observing arboreal primates in the wild and collecting faecal samples represents a challenge, especially when they live mostly on the high strata of the forest canopy and when primates adopt a cryptic behaviour. In Taï National Park, Côte d'Ivoire, several groups of

*Colobus* and *Cercopithecus* monkeys have been habituated to the presence of researchers. Ecological and behavioural studies have been conducted there since 1992 [240], and provide researchers with useful complementary information to investigate SIV infection in the context of a well known web of NHP species interaction and associations. This forest is inhabited by 9 diurnal primate species: 3 *Colobus* species, 4 *Cercopithecus* species, the sooty mangabey and the chimpanzee. [240]. Apart from sooty mangabeys, red and olive colobus, there is no information, to date, regarding the infection and the prevalence in the remaining 6 species [77, 220, 322]. SIV seropositivity has been previously described in Diana monkeys [223] and Campbell's monkeys [21], but none in lesser spot-nosed monkeys. In greater spot-nosed monkeys there are both serologic and molecular evidence of SIV infection with a prevalence of 16% among the samples collected in Cameroon [76, 79] from a different subspecies than that found in West Africa [240]. Despite extensive testing for HIV cross-reactive antibody detection, naturally occurring lentiviruses have not been detected in West African chimpanzees (*Pan troglodytes verus*) or the *P.t.vellerosus* subspecies [303, 362]. Taï chimpanzees are strongly specialised in hunting red colobus monkeys, which are infected with SIVwrc [77, 220]. Although less frequently, the other NHP species were also observed to be preyed upon by chimpanzees [32]. NHP fall prey also to the human population with the most caught monkey species in the park being the red colobus, the black & white colobus and the Diana monkey [38, 54]. In order to investigate the likelihood of West African chimpanzees and the human population to get infected with an SIV potentially acquired from NHP preys, it is important to screen these NHP species for the presence and prevalence of SIV. Serology is the "gold standard" and by using the INNOLIA HIV line assay (Innogenetics, Ghent, Belgium) more than 10 different SIV types have been so far identified [287], notably SIVwrc and SIVolc in infected blood samples [77], as well as SIVcpz and SIVgor in faecal samples of chimpanzees (*Pan troglodytes troglodytes*) and gorillas (*Gorilla gorilla gorilla*) respectively [183, 320, 386].

In this study, we report the serological and molecular results on 127 faecal samples collected from habituated free-ranging *Colobus* and *Cercopithecus* species in the Taï Forest. We then compare the relative risk for these species of being infected with SIV and the potential of cross-species transmission by considering the

ecology and behavioural data so far acquired as well as their vulnerability to human hunting.

## 9.3 Materials and methods

### Study site and samples collection

The present study was carried out in Taï National Park, on two social groups of black-and-white colobus (*Colobus polykomos polykomos*) named pol1 and pol3, on three social groups of olive colobus (*Procolobus verus*) named ver1, ver2 and ver3, on three Diana monkey groups (*Cercopithecus diana diana*), named dia1, dia2, dia3, on one group of Campbell's monkeys (*Cercopithecus campbelli campbelli*), named cam1, on one group of lesser spot-nosed monkeys (*Cercopithecus petaurista buettikoferi*), named pet1, and on a group of greater spot-nosed monkeys (*Cercopithecus nictitans stampflii*), named nic1. The park measures 4500 km$^2$ and it is located in the south-western part of Côte d'Ivoire (6°20'N to 5°10'N and 4°20W to 6°50'W). The Taï Forest represents the largest block of protected West African rainforest and is considered a biodiversity hotspot within the Upper Guinean forest region. The study site is located near the western border of the park, at about 20 km southeast of the town of Taï and it has been previously described (Figure 9.1) [358, 410].

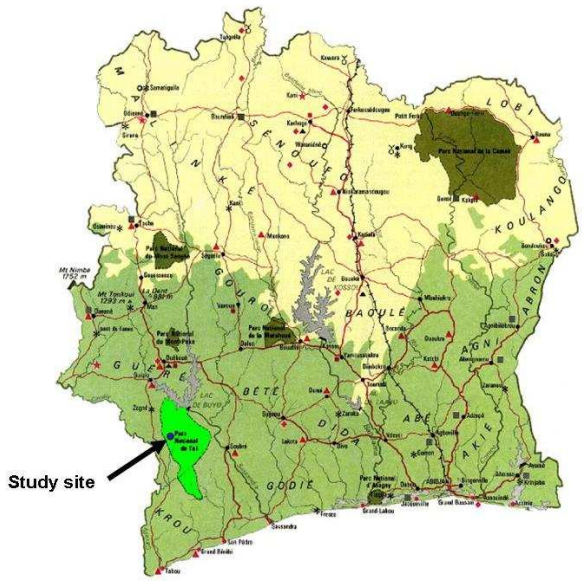



Figure 9.1: Map of Côte d'Ivoire, highlighting the geographic location of the Taï Forest (4500 km$^2$) in the south-western part of the country. Our study site is depicted with a blue dot and it is located near the western border of the park. (Revisited map based on page 63 of the Atlas de la Côte d'Ivoire, 1983, Editions Jeune Afrique-Paris).

We identified black-and-white colobus and olive colobus individuals according to facial features, pelage colour, permanent marking from fights and shape and length of the tail. Olive colobus are extremely cryptic monkeys and adult females were discriminated from males by observing prominent sexual swellings displayed during oestrus. The group size of olive colobus varies between 3 and 7 individuals. Black-and-white colobus male and female adults are quite dimorphic in size. The typical social unit of *C .polykomos* consists of one or two adult males, 3 to 7 adult females and between 6 and 12 infants, juveniles and subadults. Neighbouring groups have strongly overlapping home ranges [196]. Diana monkeys as well as Campbell's and lesser spot-nosed monkeys have also a sexually dimorphic body weight [274]. Adult females of Diana monkey were easily recognised by the presence of pendulous nipples, suggesting that they had given birth before, whereas adult males where slightly bigger than other adult individuals and they produced loud calls [98, 426, 428]. The typical Diana monkey social unit consisted of a single adult male, 6 or 7 females and their offspring [149, 274, 405]. In Taï Forest the average number of adult females was 12 individuals [50]. Campbell's monkeys and lesser spot-nosed monkeys are cryptic animals. Data culled from various reports indicate that *C. campbelli* and *C. petaurista* are generally found in small (average of 11 individuals), single male groups that exploit lower levels of the forests [46, 50, 426]. Greater spot-nosed monkeys live in groups of about 12 individuals containing a single adult male and four adult females [98]. This species is extremely rare in the Taï Forest and it has been sampled outside of the main study grid. Sampling was biased toward adults and sub-adults versus juveniles or infants, because animals were less shy and their gender was more easily identified. We collected data on group size, composition and range in parallel to the faecal sampling performed from March 2004 through July 2004. During group observation, we collected freshly dropped faecal samples (2 to 5 g) into 15 ml tubes filled with 7 ml of RNA*later* (Ambion, Austin, Texas, USA). We recorded the name of the collector, the name, sex and age class of the individual (if known) as well as the date, time and location on a painted 3 $km^2$ grid system with 100 x 100 m cells for each faecal sample (Figure 9.2). Samples were stored at camp for 30 to 60 days at 4℃ and subsequ ently shipped to the laboratory in Montpellier, France. Upon receipt samples were stored at - 80℃.

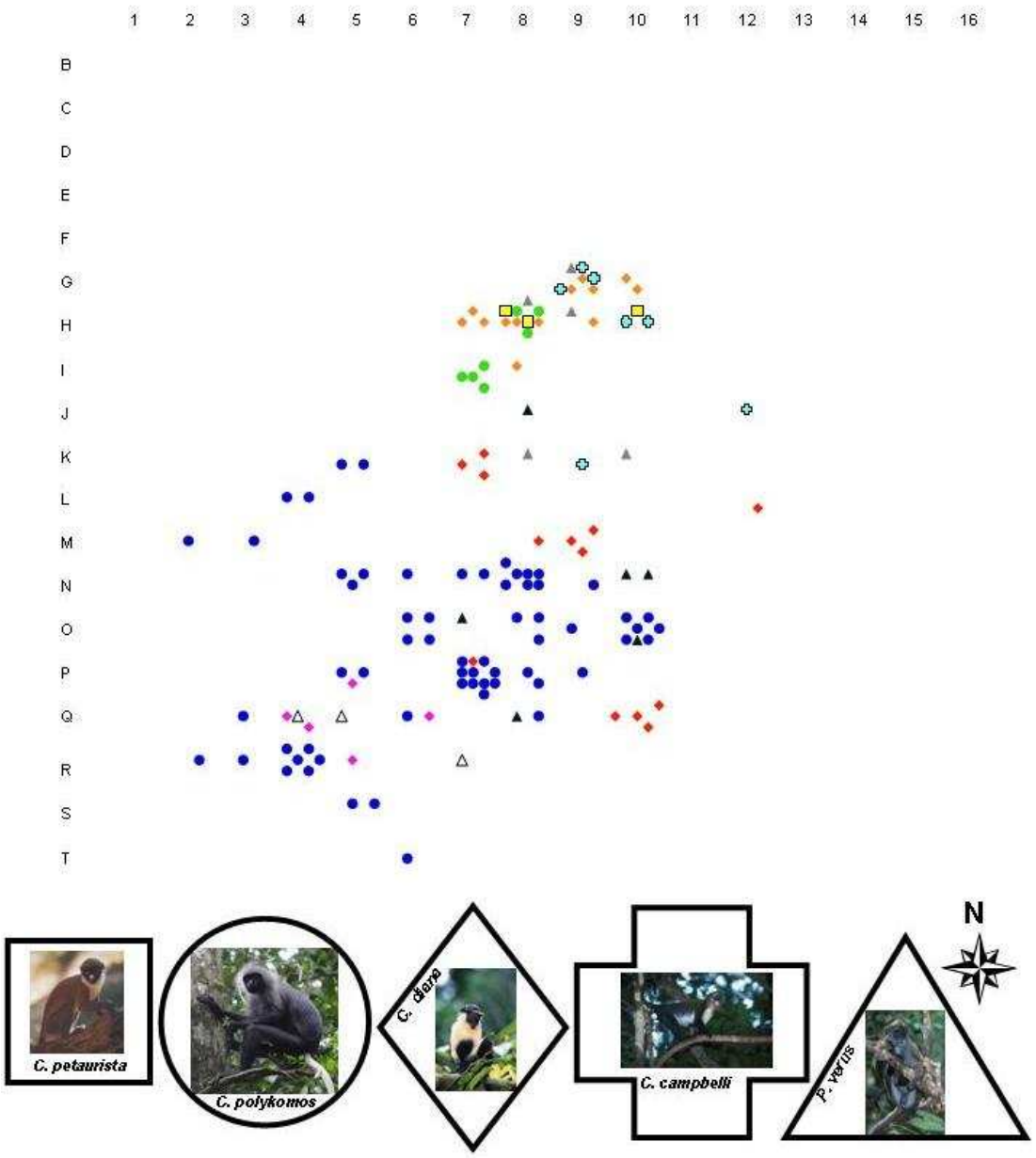



Figure 9.2: Location of faecal samples collected

Location of faecal samples collected for 2 social groups pol1 and (green dots) and pol3 (blue dots), for 3 groups of olive colobus ver1 (grey triangles), ver2 (white triangles), ver3 (black triangles), for 3 groups of Diana monkeys dia1 (red rhomboids), dia2 (orange rhomboids), dia3 (pink rhomboids), for the Campbell's monkey group (light blue crosses) and the lesser spot nosed monkeys (yellow squares) on a painted 3 $km^2$ grid system with 100 x 100 m cells. The grid system consisted of lines (trees marked in blue) in north-south direction and lines (trees marked with yellow paint) in east-west direction. Every north to south line was characterised by a letter and every east-west line by a number. Photo credits: *C.polykomos*: Sabrina Locatelli, *Procolobus verus*, *C.diana*, *C.campbelli*: Florian Möllers; *C.petaurista*: Noël Rowe.

**Detection of HIV cross-reactive antibodies in faecal samples**

We recovered IgGs after dialyses of faecal samples by applying the methods previously described for antibody detection in faecal samples of gorillas and chimpanzees [183, 386]. About 1.5 ml of faecal sample was filtered, potential viruses were inactivated and then the samples were dialysed to eliminate the presence of salt contained in the RNA*later* medium. We then tested the resulting sample for HIV cross-reactive antibodies by the INNO-LIA HIV confirmation test (Innogenetics, Ghent, Belgium). This test includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip. In addition to these HIV antigens, each strip has control lines: one sample addition line (3+) containing anti-human immunoglobulin (IgG) and two test performance lines (1+ and +/-) containing human IgG. We performed all assays according to manufacturer's instructions. Samples were scored as INNO-LIA positive when they reacted with at least one HIV antigen and had a band intensity equal to or greater than the assay cutoff (+/-) lane; samples that reacted less strongly, but still visibly with two or more HIV antigens were classified as indeterminate; and samples reacting with no bands or only one band with less than +/- intensity were classified as negative.

**Nucleic acid extraction from faecal samples**

We extracted viral RNA from faecal samples using the RNAqueous-Midi kit (Ambion, Austin, Texas, USA) as previously described [183, 321]. Briefly, 6 ml of lysis-binding solution was added to 1.5 ml of faecal sample solution and vortexed vigorously until the sample was thoroughly homogenised. The suspension was clarified by centrifugation (at 4500 RPM for 5 min) and an equal volume of 64% ethanol was added. The solution was passed through a glass fibre filter unit to bind nucleic acids and washed three times with washing buffer. The nucleic acids were then eluted (1200 µl) and subsequently precipitated with LiCl and spun at 13000 RPM. The resulting pellet was washed once with cold 70% ethanol, air dried, re-suspended in 50 µl of RNase free-water and then stored at -80℃. We extracted faecal DNA using the QIAamp Stool DNA mini kit (QIAGEN, Hilden, Germany). Briefly, 2 ml of faecal RNA*later* mixture were centrifuged and the pellet was re-suspended in stool lysis buffer, clarified and passed through a DNA binding column. Bound DNA was eluted in 100 µl storage solution and stored at -20℃.

**Amplification of SIV from faecal RNA**

We performed RT-PCRs on faecal virion and PCRs of integrated genomic DNA using species specific as well as universal primers: the primers sequences, annealing temperatures, amplicon sizes, targeted region of the SIV genome and references are listed in Table 9.1. PCR amplifications were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Manheim, Germany) according to the manufacturer's instructions. Each amplification reaction included a manual hot-start at 94℃ followed by 35 cycles with a denaturation step at 94℃ for 20sec, an annealing temperature set according to the primer melting temperatures and a variable extension time depending on the size of the expected fragment (1mn/kb). PCR products were purified (Q-Biogene, Illkirch, France), and directly sequenced using the inner primers on an ABI 3130xl Genetic Analyser (Applied Biosystem, Courtaboeuf, France). We then checked and assembled the sequences using the software package Lasergene (DNASTAR Inc. Madison, USA).

**Microsatellite analyses**

In order to confirm the origin of faecal samples collected, we performed microsatellite analyses on all samples collected from the forest floor, except for chimpanzees samples which were clearly taken from different carcasses. We targeted the following microsatellite loci: D3s1768, D4s243, D5s1475, D6S501, D7s503, D17s791. They have originally been isolated from human DNA and were found to yield amplification products in several primate species [72, 135]. The amplification reactions as well as the multiplex PCRS procedures are described elsewhere [219]. Amplification products were separated using capillary electrophoresis on a ABI 3100 genetic analyzer (Applied Biosystems, UK). Alleles were sized relative to an internal size standard (ROXHD400) using Gene Scan 3.7 (Perkin-Elmer Applied Biosystems, UK). We followed a multi-tube approach whenever possible, with at least seven independent positive PCR reactions confirming a homozygote and at least three reactions confirming both bands of a heterozygote [115, 253, 363], to prevent incorrect genotypes due to stochastic amplification of only one of two possible alleles (allelic dropout). We relied on a few high-quality DNA samples as a species control, mostly to verify whether the two sources had comparable allele sizes, and we also extracted DNA from hair from two

researchers involved in this study and genotyped at each locus to detect possible human contamination at both the sample collection and PCR stage [4, 395].

**Qualitative analysis of behavioural and ecological data and primate vulnerability to human hunting pressure**

In order to provide an additional dimension to the multifaceted aspects of SIV transmission, we focused on previously collected behavioural and ecological data, which could represent potential risk factors of SIV transmission in NHPs and we also assessed their vulnerability to human hunting pressure. We looked at (1) the group size of a social unit, as this value would influence the probability of being next to an individual carrying the virus, if this virus would be randomly distributed. (2) The number of offspring a female can have, given that SIV could be vertically transmitted. This number depends on the average lifespan of the mother, the age reached at sexual maturity and on the inter-birth interval. (3) The adult sex ratio and the mating system, which will determine the number of potential sexual partners. (4) The population density, territorial behaviour and feeding ecology as factors determining both inter- and intra-group competition for food or mate resources as well as inter-species competition and predation. (5)The likelihood of contact with bleeding wounds occurring during grooming activity, performed with the fingers or directly with the mouth by another member of the group. (6) The gender who transfers at sexual maturity and the frequency of social group change. (7) The frequency of mixed species associations, another potential source of SIV spread between species, if affiliative or agonistic behaviour occurs.

We evaluated the risk factors of virus transmission from NHPs to the human population, by gathering information on the extent of harvested animals. We estimated the primate species vulnerability to hunting pressure by considering population density, hunting pressure, adaptation and forest stratum use. We also gathered information on detention of NHPs as pets, animal parts used as fetiches, taboos and crop-riding behaviour.

## 9.4 Results

### Samples and animal identification

We collected a total of 127 faecal samples from *Colobus* and *Cercopithecus* monkeys between March and July 2004. The number of samples collected per species investigated is listed in Table 9.2.

Table 9.2: Species investigated, number of samples collected and analysed

| Species | Group | Nbr of samples collected | Nbr samples msats analysis | Nbr samples successfully genotyped | Nbr individ. | Nbr samples LIA analysis | Nbr samples vRNA analysis | Nbr samples G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|
| ***Colobus polykomos*** | Pol1 | 7 | 6 | 6 | 6 | 4 | 5 | 2 |
| | Pol3 | 63 | 28 | 23 | 15 | 53 | 27 | 7 |
| ***Procolobus verus*** | Ver1 | 5 | 4 | 3 | 3 | 4 | 3 | 5 |
| | Ver2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 |
| | Ver3 | 6 | 6 | 6 | 4 | 5 | 5 | 1 |
| ***Cercopithecus diana*** | Dia1 | 13 | 13 | 13 | 11 | 13 | 13 | 2 |
| | Dia2 | 13 | 13 | 12 | 9 | 13 | 12 | 0 |
| | Dia3 | 5 | 5 | 5 | 3 | 15 | 3 | 0 |
| ***Cercopithecus campbelli*** | Cam1 | 7 | 7 | 7 | 6 | 7 | 1 | 7 |
| ***Cercopithecus petaurista*** | Pet1 | 3 | 3 | 3 | 3 | 2 | 1 | 2 |
| ***Cercopithecus nictitans*** | Nic1 | 2 | 2 | 2 | 2 | 2 | 1 | 2 |

[1]Pol1; Pol3: *C.polykomos* group 1and group 3; Ver1, Ver2, Ver3: *P.verus* group 1, group 2, and group 3; Dia1, Dia2, Dia3: *C.diana* group 1, group 2, and group 3; Cam1: *C.campbelli* group1; Pet1: *C.petaurista* group 1; Nic1: *C.nictitans* group 1.

[2]Nbr of samples successfully genotyped: at least three loci provided reliable and repeatable results.

Table 9.3 shows the percentage of sample coverage (going from 25% to full coverage) of the adult population of all social groups selected, considering the group census effectuated in 1999 for the colobus monkeys [191] and in 2001 for the guenons [50].

Table 9.3: Percentage of sample coverage of the adult population of censused social groups.

| | census 1 (1999)[a] census 2 (2001)[b] | | Samples collected Mach-July 2004[c] | | | |
|---|---|---|---|---|---|---|
| **Group** | **Adult male** | **Adult female** | **Adult male** | **Adult female** | **Adult nd sex** | **% of the adult pop sampled** |
| **B&W colobus** | | | | | | |
| Pol1 | 1-2 | 4-6 | - | - | 6 | 75% |
| Pol3 | 1 | 6 | 2 | 8 | 4 | > 100% |
| **olive colobus** | | | | | | |
| Ver1 | 1-2 | 1-3 | 2 | 1 | - | 60% |
| Ver2 | nd | nd | - | 2 | 1 | na |
| Ver3 | 1 | 1-2 | 2 | 2 | - | > 100% |
| **Diana monkey** | | | | | | |
| Dia1 | 1 | 11 | - | 3 | 7 | 83% |
| Dia2 | 1 | 13 | 1 | 6 | 2 | 64% |
| Dia3 | 1 | 11 | - | 1 | 2 | 25% |
| **Campbell's monkey** | | | | | | |
| Cam1 | 1 | 6 | - | 3 | 3 | 86% |
| **Lesser spot-nosed monkeys** | | | | | | |
| Pet1 | 1 | 8 | 1 | 1 | 1 | 33% |
| **Gretaer spot-nosed monkeys** | | | | | | |
| Nic1 | 1 | 4 | - | 1 | 1 | 40% |

[a]census of colobus monkeys [191]; [b]census of the guenons [50]; [c] excluding unscorable results of microsatellite analysis; nbr of individuals according to microsatellite analysis and not to observed individuals; nd: not determined; na: non applicable[d]

Several individuals from the black-and-white and the olive colobus groups were visually recognised, for the *Cercopithecus* species only the sex and the age were clearly assessed. We therefore performed microsatellite analysis for samples discrimination and definitive individual identification. To date, there are no published microsatellite data for the western black-and-white and the olive colobus, but a few loci have been identified for the Diana monkeys [72] and the greater spot-nosed monkeys [35]. We chose six highly polymorphic markers (D3s1768, D4s243, D5s1475, D6s501, D7s503, D17s791) from a panel of nine human microsatellite loci, which were found to yield amplification products in several primate species and which loci were informative enough to exclude sample misidentification (Locatelli et

al. submitted.). The number of successfully genotyped samples as well as the number of resulting individuals is summarized in Table 9.2. Microsatellite analysis results including amplicon sizes for each of the loci are listed in Table 9.4.

**Detection of HIV cross-reactive antibodies**

Blood samples from red and olive colobus previously identified using the INNOLIA-HIV confirmation assay showed clear IgG bands and cross-reacted with HIV antigens for the core protein p24, the matrix protein p17 or the envelope glycoproteine gp36 [77]. Consequently, and in analogy with studies on wild living chimpanzees and gorillas, we also tested 112 out of 127 faecal samples belonging to *Colobus* and *Cercopithecus* species (Table 9.2) The remaining samples were not considered because of insufficient material availability, unsuitable storage conditions or degraded material. For the black-and-white colobus 42 samples revealed a clear presence of IgG, but did not react with any of the HIV antigens. The 15 remaining samples had to be considered ‘not interpretable’, since the baseline results did not fit the requirements, i.e., the anti human IgG upper line was absent or gave only a weaker signal than that of the lower human IgG line on the strips, and no reactivity with any HIV antigens was observed (Table 9.4). For the olive colobus, only 2 samples revealed a clear presence of IgG, but did not react with any of the HIV antigens. The 11 remaining analysed samples had to be considered ‘not interpretable’, for the same reasons listed above. For the *Cercopithecus* species the 42 samples analysed revealed a clear presence of IgG, but none of them reacted with any of the HIV antigens and were therefore considered to be negative.

**Detection of SIV infection in Taï primate communities by amplification of viral RNA in faeces**

We extracted RNA from 78 faecal samples belonging to at least 53 individuals. Details on the number of samples and corresponding individuals analysed are given in Tables 9.2 and 9.4. The second last and the last column of Table 9.4 provide information on the outcome of faecal SIV RNA detection and on PCR amplification of ~1500 bp in the region of the glucose-6-phosphate dehydrogenase gene (G6PDH) and a ~390 bp fragment of the mitochondrial DNA spanning the 12SrRNA gene. These latter PCRs were performed on a limited set of samples to cross-check for DNA quality (the ability of scoring alleles by microsatellite

analysis is already a good indicator of host DNA quality). The numbers written in parenthesis next to the PCR results refer to the primer sets used, listed in detail in Table 9.1.

Consensus primers amplifying fragments ranging from ~200 to ~800 bp of the SIV *pol* gene, which we tested on samples from all species, gave no positive result. We also used more specific primers amplifying fragments of the *pol* gene in the *Cercopithecus* monkeys [1], which we tested on the Diana, the Campbell's, the lesser and greater spot-nosed monkeys. Here as well, we did not obtain any positive result. Specific primers amplifying SIVwrc *pol* and *env* fragments of 650 and 570 bp respectively [219], were tested on *Colobus polykomos* and on *Procolobus verus*, based on the hypothesis that SIV would have coevolved within the subfamily of the *Colobinae*. Additionally newly designed primers (olcpol) targeting a smaller region of *pol* of about 300 bp were also tested on olive colobus, given their suspected RNA degradation. None of these sets of primers did amplify the targeted fragment, neither in the black and white nor in the olive colobus.

Another set of consensus degenerate primers targeting a fragment of 252bp in the *gag* region of SIVwrc [218] was tested on black and-white colobus, Diana monkeys and the chimpanzees with no positive outcome. Some black and white faecal samples were also tested with primers amplifying SIVcol from a tissue sample of mantled guereza (*Colobus guereza*) from Cameroon whose genome has been fully sequenced [78]. We designed additional primers (cgzpol and bwcpol) based on the alignment of consensus sequences of SIVcolCGU1, SIVwrc-98CI-04, SIVwrc-97CI-14 and SIVolc-97CI-12. They amplify a fragment of ~500 and ~600 bp respectively. These primers were tested on the western black-and-white species, based on the hypothesis that the western and the west-central *colobus* species could harbour an SIV, which coevolved together with this genus or possibly with the subfamily of the *Colobinae*. According to the results obtained, all the black-and-white colobus faecal samples tested were SIV negative. All PCRs performed included tissue samples from red colobus, olive colobus, or mantled guereza, which were previously identified as SIV positive.

**Socio-ecological characteristics of the NHP species inhabiting the Taï Forest**

Table 9.5 presents ecological and behavioural data on the species investigated, which have been collected from different studies conducted not only in the Taï Forest, but also in other forests of West Africa. Data on the sympatric sooty mangabeys and chimpanzees are included for comparison.

If we consider exclusively the group size and if we assume SIV as randomly distributed, red colobus, sooty mangabeys or chimpanzees would be the best candidates for its spread. Supposing for example, that the frequency of infection in a population is 20%, there will be 8 chimpanzees infected in a group of 40 individuals, 14 individuals in a group of 70 sooty mangabeys and only one to two individuals in a group of 7 olive colobus.

However, group size is not the only factor that matters. SIV is a sexually transmitted virus, therefore the mating system of a species and the adult male/female ratio play a role in the pattern and extent of SIV transmission. Red colobus monkeys, sooty mangabeys and chimpanzees have the highest adult male/female ratio, but their mating system is different. Red and olive colobus live in promiscuous groups where there is no competition for mates and no established hierarchy. In olive colobus females have been observed to mate with male group members and with extra-group males as well [195]. Male sexual coercion can also occur, and it increases with the relative number of adult males in a group [191]. Black-and-white colobus live in a polygynous society and forest guenons typically live in single-male groups for most of the year. During the breeding season, single male groups are sometimes invaded by non-resident males who may stay for several days to months and occasionally manage to expel the resident male [50, 75]. To a certain degree promiscuity offers less of a social barrier to pathogens, therefore we can speculate that red and olive colobus would be the species facing the highest chance of giving or receiving a sexually transmissible disease.

The likelihood and the frequency of vertical transmission of SIV from mother to offspring *in utero* have not been extensively studied. However, if we consider this route of transmission possible, then the species where females reach sexual maturity at an early life stage, and where birth intervals are short, will give birth to more offspring in their life time, thus increasing the chances of vertically transmitting the virus.

The data gathered on the average life span of the different species are substantially similar, therefore the *Colobu*s, *Cercopithecus* or C*ercocebus* females, whose inter-birth interval varies between one and two years, will potentially transmit the virus many more times to their offspring than the chimpanzees, whose interval averages 5 to 6 years.

When the offspring reaches sexual maturity, one or both genders will be ready to migrate to another social group to avoid inbreeding depression. The degree of SIV vertical transmission will determine the percentage of infected versus non infected individuals among the sexually immature population. The gender which disperses would be responsible for the transmission of SIV to naïve individuals from another social group.

Within the same line of reasoning, if promiscuity is the condition which creates the highest number of infected individuals and therefore the highest number of infected mothers and potentially the highest number of infected offspring, then red colobus would be one of the most efficient SIV transmitters, via female biased dispersal. Olive colobus would be even more successful, as females and males appear to leave quite readily and regularly even after breeding in a group [195]. In the chimpanzee society the males are philopatric, with females leaving the natal group. In black-and-white colobus the dispersal is male biased, more prominently so in the guenons and in sooty mangabeys. These societies do not allow a promiscuous behaviour, but rank order or sexual supremacy are dynamic patterns, which over time would permit SIV transmission, unless this virus would affect somehow the fertility of an individual.

SIV transmission could also occur via blood contact through aggressive bites between members of the same or of a different group or species or via affiliative behaviour, by grooming or licking of open wounds. The degree of exposure to these behaviours will depend on the level of competition, which in males is mainly related to mating opportunities, and in females is related to food and safe places [378].

Competition for food is defined by two extremes: scramble and contest competition. Scramble competition prevails when resources are evenly distributed in nature, in the form of small items and consequently fighting over a resource is not worth the energy and risk of injury (ex.: the colobus species which are mainly folivourous). Contest competition prevails when an individual that monopolises a resource increases its reproductive success over that of its competitors. Therefore,

contest competition is expected when resources are distributed in small but rich patches set wide apart (ex: frugivorous or omnivorous species like the guenons, sooty mangabeys and the chimpanzees) [388, 391]. When competition is strong, individuals may enhance their competitive strength by forming coalitions or alliances (as for chimpanzees [32] and affiliative interactions would hold groups and coalitions or alliances together and ease tension arising from intra-group competition.

Based on the socio-ecological theory and taking into account a very general view of the species studied, we can speculate that food competition would be less intense in the colobus species than in the guenon, the mangabeys or the chimpanzees. Except for the black-and white colobus, all the other species are considered territorial and the males are normally in charge of territory defence. Competition for mates would be more intense in multi-male societies, regulated by a rank system as in sooty mangabeys and in chimpanzees. Inter-groups aggressive behaviour would be more intense in populations living at high densities, as in the case of red colobus and Diana monkeys. In fact, Diana monkeys live in consistently larger groups and showed higher rates of inter-group aggression and intra-group agonistic behaviour in relation to the other guenon species [50].

All primates species present in Taï, except the chimpanzee, are either folivores or frugivores - insectivores species. Chimpanzees are omnivores. Hence, the chimpanzee is the only likely species to get in contact with a virus by handling and consuming the flesh of potentially infected animals.

Finally, the association of different monkey species as an anti-predation strategy against chimpanzees but also leopards (*Panthera pardus*) or crowned eagles (*Stephanoaetus coronatus*) provides occasions for social interactions between different species (i.e. grooming has been observed). Diana monkeys are the preferred species to associate with, because of their ability to detect earlier than other species the presence of a predator, thus representing the best candidate for the exchange and spread of viruses.

**Interactions between humans and NHPs**

In Côte d'Ivoire, hunting is prohibited throughout the country. Nevertheless it is commonly practiced [55]. The availability of livestock meat is very low in rural areas of Taï [54], so bushmeat is the daily food of the rural people. The market surveys indicate that in general larger preys are preferred because of the higher revenue per

shot: hunting pressure being highest on the red colobus and the mangabeys in the eastern part, whereas the black and white colobus and the red colobus are most affected in the western part of the Taï area [54]. Most hunting of primates is carried out with non-traditional weapons (i.e. shotguns) and is done on a large (non-subsistence) scale [310].

The vulnerability to hunting pressure of the Taï Forest monkeys varies according to their body size, their substrate preference (terrestrial or arboreal), their response to humans and their density. Large body size, large group size, noisy behaviour, and reluctance to flee from hunters, make red colobus a favourite prey for poachers. Diana monkeys are almost equally vulnerable, as they are the noisiest and most raucous monkeys in the Taï Forest. However, in some regions, they have adapted their behaviour in response to human hunting pressure, contrary to red colobus [38]. Black and white colobus are probably hunted less often than red colobus because they live in smaller, more cryptic societies and at a lower density [191]. Similarly, the habitat use, the coloration, their cryptic behaviour and the low population density makes the olive colobus a difficult species to locate and kill. Campbell's monkeys live in small, cryptic groups. They are probably more difficult to hunt than Diana monkeys or red colobus. However, the fact that this species is able to exploit areas of disturbed forest puts them in closer proximity to humans, where they are killed near villages or agricultural fields [238]. In many ways, the lesser spot-nosed monkey is ecologically similar to Campbell's monkey and the factors responsible for their vulnerability are the same. The ability of this monkey to exploit degraded forest near villages makes it an easy target for hunters and it is one of the most common monkeys found in bushmeat markets [240].

In many regions of West Africa primates are not consumed, because it is forbidden by the Islamic religion and certain animist tribes consider chimpanzees as totems [189]. Taboos concerning primate meat are tribe or family specific in the Taï region. Hence, with the mixture of people from different origin and different cultures, the importance of taboos for conservation decreases. Migrants often kill species that are considered taboo by local people and vice versa.

There is no data available on crop raiding behaviour around the Taï area, and on pet trade either, although captive sooty mangabeys, infant chimpanzees, Diana

and Campbell's monkeys have been seen in villages around the Park and bites from chimpanzees have been reported (Emmanuelle Normand, personal communication).

## 9.5 Discussion

**Black and white and olive colobus**

The INNO-LIA HIV I/II confirmation assay detected SIVwrc and SIVolc from western red colobus and olive colobus blood samples [77] as well as SIVcpz and SIVgor from West-central chimpanzees and West-central gorillas [183, 386]. However, when western red colobus faecal samples were tested, IgGs were absent or present only in low quantities. The results on the strips were therefore impossible to interpret [219]. We observed the same profiles in olive colobus faecal samples. Only two samples out of 13 could be reliably interpreted. We know from previous work that olive colobus are infected with SIVolc [77], but the prevalence of infection in the wild is not known. The PCRs performed on olive colobus from three neighbouring groups were all negative. There are at least three plausible explanations: (1) the faecal material was already partly degraded, (2) the primers designed were not specific enough to amplify additional olive colobus samples, (3) the individuals analysed are not infected with SIV. This seems unlikely, given the social structure they live in (allowing migration of both sexes) and their promiscuous mating system (increasing the chances of sexually transmitted viruses). We have obtained a higher percentage of interpretable serological results in black and white compared to olive colobus (74% versus 15%). The majority of samples providing non interpretable results belong to a first set of samples collected early in the field season, suggesting a short time frame for effective serological analysis. Neither RT-PCRs using specific primers amplifying SIVcol in the West-central mantled guereza nor consensus primers for different species of the Colobinae subfamily (SIVwrc, SIVolc, SIVcol) amplified SIV in the western black-and-white colobus. Here again there are four possible reasons for these negative results: (1) mantled guereza and western black-and-white colobus belong to the *Colobus* genus of the Colobinae subfamily, but they are two different species, inhabiting different regions of Africa. Analysis of loud calls, cranial morphology and pelage indicate that western black-

and-white colobus diverged early from the ancestral black and white colobus, while *C. vellerosus* and *C.guereza* to the east are more recently derived forms [271, 273]. If SIVcol has not coevolved with this genus, but has infected colobus once host speciation has occurred, then *Colobus polykomos*, living today in the upper Guinean forests, could have been spared from being infected with SIV. (2) *Colobus polykomos* may carry a species specific SIV, but the primers developed may not be specific enough to amplify the virus. (3) As for the bwcpol primers, the region targeted in the first round of PCR may be too big for partially degraded faecal samples to enable a successful amplification. (4) *Colobus polykomos* live in a polygynous society (table 9.5), therefore we cannot exclude that, at a given time, SIV prevalence could be inexistent in certain communities and much higher in others.

***Cercopithecus* monkeys**

This is the first time that faecal samples from *Cercopithecus* monkeys have been tested with the INNO-LIA HIV confirmation assay. In contrast to what we observed for the *Colobus* species, the *Cercopithecus* monkeys' faecal samples tested with the INNO-LIA assay satisfied the baseline requirements for the test validation, but all samples resulted negative. Regardless of the date of the sample collection and of the repeated freeze and thaw procedures, we have observed that antibody titers in faeces could also vary according to the host species investigated and could be related to the host's different digestive physiology and diet composition. *Cercopithecus* monkeys forage on fruits, invertebrates and leaves, their strategy of food storage relying on the presence of cheeck pouches. The fact that, contrary to colobines, *Cercopithecus* monkeys are not able to process relatively indigestible food and that they are not able to evade digestion inhibitors and toxins, could account for a different gastro-intestinal microflora, which would allow antibody titers to be more easily detected. To corroborate this speculation, we were able to obtain more reliable results in black and white colobus, which are the least folivorous of the colobines [191, 192], compared to olive colobus (this study) and western red colobus [219]. Another aspect to consider is that, apart for *C.cephus* and *C.mona*, whiose sera were found to be SIV positive using INNO-LIA confirmation tests [76], we do not know if other *Cercopithecus* species would react positively using the same assay. SIV antibody detection using synthetic peptides would be indeed more specific [2], but

the limitations encountered so far are related to the lack of sensitivity and appropriate standardisation in faecal samples.

If we assume the INNO-LIA results to be reliable and the PCRs performed targeting the gene regions selected, only the dia3 group would be considered to have been fully sampled, together with the Campbell's monkey group cam1 (applicable only for the INNO-LIA test) assuming a 20% prevalence in every group, regardless of the species (Table 9.3). In general, more samples should be collected to reach a better coverage of the adult population per group and the collection should also be extended to more than one or two neighbouring groups. Ideally, INNO-LIA assays should be performed on site, to prevent further sample degradation due to thawing and re-freezing procedures and delayed laboratory analysis.

No new SIV infections are reported in this study. The significance of the behavioural and ecological data gathered can nonetheless help us determining the likelihood of a species to be infected with SIV and to better understand SIV spread dynamic among primate populations. From the behavioural and ecological information gathered on 9 primate species, red colobus, olive colobus, Diana monkeys, sooty mangabeys and chimpanzees seem to be the species more prone to be infected with SIV. Red and olive colobus should be the species with the highest prevalence of infection, given their promiscuous mating system. So far, positive results have been obtained in sooty mangabeys, whose prevalence in the wild was estimated at 59% [322], whereas in red colobus, SIVwrc was estimated to infect at least 26% of the adult population [219].

Additionally, regardless of the SIV positivity and prevalence, quantitative and qualitative estimations of risk factors suggest that humans are more at risk of pathogens exposure (possibly including SIV) of red colobus, Diana monkeys, sooty mangabeys and chimpanzees in comparison to the remaining species. Taking everything into consideration, we should focus our efforts to isolate SIVs in Diana monkeys and West African chimpanzees. Moreover, the human population surrounding the Taï National Park is constantly increasing [185]. With an easier access to remote forest areas and the professionalization of the wild meat trade, bushmeat will become more and more available in urban areas as well [54]; consequently, the risk of viral cross-species transmission will not diminish.

## 9.6 Acknowledgments

We thank the ‘Ministère d’Enseignement Supérieur et Recherche Scientifique’, the ‘Ministère d’Agriculture et Ressources Animales’, the ‘Centre Suisse de Recherche Scientifiques’(CSRS) in Abidjan, the ‘P.A.C.P.N.T.’ and the ‘Centre de Recherche en Ecologie’ in Côte d’Ivoire for support and permission to conduct research in the Taï National Park. We thank the Taï Monkey Project and in particular Ferdinand Bélé, Cécile Benetton and Bertin Diero, for helping out in samples collection; Christelle Butel, Fran Van Heuverswyn and Nicole Vidal for technical advice in the laboratory in Montpellier; Fabian Leendertz and Johannes Refisch, for involvement in the early phase of the project, Marcel Tanner and Jakob Zinsstag for critical support outside the field. The virology section of this study was financially supported by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de Recherches pour le SIDA (ANRS).

Sabrina Locatelli was supported by grants from the Commission for Research Partnerships with Developing Countries (KFPE), Bern, Switzerland, to conduct field research and by the Messerli foundation, Zürich and the Guggenheim-Schnurr Foundation, Basel, Switzerland, to conduct laboratory analyses on the monkey genetics.

Table 9.1: Species investigated and details on primers tested

| Species | Primers tested | Sequences | TM (%GC) (℃) | Estimated amplicon size | Region targeted | Reference |
|---|---|---|---|---|---|---|
| ***C. polykomos*** | DR1-DR2/DR4-DR5 (1) | DR1 (5'-TRCAYACAGGRGCWGAYGA-3') | 58 | 800 | *pol* | [67] |
| | | DR2 (5'-AIADRTCATCCATRTAYTG -3') | 42.7 | | | |
| | | DR4 (5'-GGIATWCCICAYCCDGCAGG-3') | 60 | 200 | | |
| | | DR5 (5'-GGIGAYCCYTTCCAYCCYTGHGG -3') | 64 | | | |
| | polOR-polis4/polis2-uni2 (2) | polOR(5'-ACBACYGCNCCTTCHCCTTTC -3') | 53.1 | 800 | *pol* | [78] |
| | | polis4(5'-CCAGCNCACAAAGGNATAGGAGG-3') | 55.2 | | | |
| | | polis2(5'-TGGCARATRGAYTGYACNCAYNTRGAA-3') | 54.7 | 650 | | |
| | | uni2(5'-CCCCTATTCCTCCCCTTCTTTTAAAA -3') | 53.3 | | | |
| | wrcpol (3) | wrcpolF1 ( 5'-TAGGGACAGAAAGTATAGTAATHTGG-3') | 50.9 | 1100 | *pol* | [220] |
| | | wrcpolR1 (5'-GCCATWGCYAA TGCTGTTTC-3') | 49.7 | | | |
| | | wrcpolF2 (5'AGAGACAGTAAGGAAGGGAAAGCAGG-3') | 54.4 | 650 | | |
| | | wrcpolR2 (5'-GTTCWATTCCTAACCACCAGCADA-3') | 51.4 | | | |
| | wrcenv (4) | wrcenvF1 (5'-TGGC AGTGGGACAAAAATATAAAC-3') | 50 | 750 | *env* | [220] |
| | | wrcenvR1 (5'-CTGGCAGTCCCTCTTCCA AGTT GT-3') | 55.3 | | | |
| | | wrcenvF2 (5'TGATAGGGMTGGCTCCTGGTGATG3') | 56.6 | 550 | | |
| | | wrcenvR2 (5'-AATCCCCATTTYAACCAGTTCCA-3') | 51.1 | | | |
| | wrcgag (5) | wrcgagF1 (5'-ATDGAGGATAGAGGNTTTGGAGC-3') | 50.2 | 600 | *gag* | [218] |
| | | wrcgagR1 (5'-GCCCTCCTACTCCTTGACATGC-3') | 53.4 | | | |
| | | wrcgagF2 (5'-CCAACAGGGTCAGATATAGCAG-3') | 49.7 | 250 | | |
| | | wrcgagR2 (5'-ACTTCTGGGGCTCCTTGTTCTGCTC-3') | 55.9 | | | |
| | olcpol (6) | | | | | |
| | cgzpol (7) | cgzpolF1 (5'-CAGTGYTGGATATAGGAGATGCC-3') | 51.9 | 700 | *pol* | unpublished |
| | | cgzpolR1 (5'-ACTGCATAGCCCCATTGTCC-3') | 48.7 | | | |
| | | cgzpol F2 (5'-CAGCTTTYACAGTGCCATCAGTG-3') | 51.9 | 500 | | |
| | | cgzpolR2 (5'-TCTTCTGCTTCTGCACTAAGCTG-3') | 50.2 | | | |
| | bwcpol (8) | bwcpolF1 (5'-TAGATACAGGAGCAGATGATACAGT-3') | 49.3 | 1000 | *pol* | unpublished |
| | | bwcpolR1 (5'-ATTDCCYCCTATCCCTTTATGWGC-3') | 52.7 | | | |
| | | bwcpolF2 (5'-AGAYTRGAAGCAGARGGAAAAAT-3') | 44.8 | 600 | | |
| | | bwcpolR2 (5'-TCCYACCAATTTYTGTAYATCATTTACTGT-3') | 51.1 | | | |

Table 9.1: Species investigated and details on primers tested (continued)

| Species | Primers tested | Sequences | TM(%GC) (℃) | Estimated amplicon size | Region targeted | Reference |
|---|---|---|---|---|---|---|
| ***P. verus*** | wrcpol (3) | | | | | |
| | wrcenv (4) | | | | | |
| | olcpol (6) | olcpolF1(5-TAGATACAGGRGCAGATGAYACAGTAAT-3') | 53.8 | 700 | *pol* | unpublished |
| | | olcpolR1 (5'TCCAYCCYTGAGGHARYACATTATA-3') | 47.7 | | | |
| | | olcpolF2 (5'-CTAGAATWATWGGRGGRATAGGRGG-3') | 52.6 | 300 | | |
| | | olcpolR2 (5'-ATYTTWCCTTCTKCTTCYARTCTRTCACA-3') | 50.8 | | | |
| ***C. diana*** | DR1-DR2/DR4-DR5 (1) | | | | | |
| | CNMF1/POLor2/ CNMF2/CNMR2 (9) | | | | | |
| | polOR-polis4/polis2uni2 (2) | | | | | |
| | wrcgag (5) | | | | | |
| ***C. campbelli*** | CNMF1/POLOR2/ CNMF2/CNMR2 (9) | CNMF1(5'-TATCCYTCCYTGTCATCYCTCTTT -3') | 51.35 | 2750 | *pol* | [1] |
| | | POLOR2(5'-ACBACWGCTCCTTCWCCTTTCCA -3') | 70 | | | |
| | | CNMF2 (5'- AATGGAGAATGYTMATAGATTTCAG-3') | 49.4 | 2050 | | |
| | | CNMR2 (5'-CCCCYATTCCTCCCTTTTTTTTA -3') | 51.07 | | | |
| | DR1-DR2/DR4-DR5 (1) | | | | | |
| | polOR-polis4/ polis2uni2 (2) | | | | | |
| ***C. petaurista*** | CNMF1/POLor2/ CNMF2/CNMR2 (9) | | | | | |
| | DR1-DR2/DR4-DR5 (1) | | | | | |
| | polOR-polis4/ polis2uni2 (2) | | | | | |
| ***C. nictitans*** | CNMF1/POLor2/ CNMF2/CNMR2 (9) | | | | | |
| | DR1-DR2/DR4-DR5 (1) | | | | | |
| | polOR-polis4/ polis2uni2 (2) | | | | | |

Y=C/T, W=A/T, R=A/G, H=A/C/T, B=C/G/T, S=G/C, K=G/T, D=A/G/T, N=A/C/T/G Y=C/T, M=A/C, K=G

Table 9.4: Genotype results at 6 loci and SIV results for all samples collected

| [a]Faecal spl. | Social group | [b]Sex/age | Indiv. name | [c]locus D3s1768 | locus D4s243 | locus D5s1457 | locus D6s501 | locus D7s503 | locus D17s791 | [d]indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 19 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 20 | Pol3 | nd/A | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 21 | Pol3 | nd/A | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 23 | Pol3 | nd/A | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 24 | Pol3 | M/SA | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 40b | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 41 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 42 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 43 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 44 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 98 | Pol3 | F/A | Saly | ns | 133145 | 102110 | 131139 | 146156 | 157165 | 11 | neg | neg (1, 2, 7) | neg/nd |
| 99 | Pol3 | M/A | Koly | nd | nd | nd | nd | nd | nd | nd | neg | neg (1, 2, 5, 7) | nd |
| 100 | Pol3 | F/A | Sea | ns | ns | 110114 | ns | 154156 | 157165 | 16 | neg | neg (5) | nd |
| 101 | Pol3 | F/A | Rita | 153157 | 145153 | 110114 | 131139 | 144146 | 157163 | 18 | nd | nd | nd |
| 104 | Pol3 | F/A | Sea | ns | ns | 110114 | ns | 154156 | 157165 | 16 | neg | neg (1, 2, 3, 4, 5, 7) | nd |
| 105 | Pol3 | F/A | Rita | 153157 | 145153 | 106110 | 131139 | 144146 | 157165 | 19 | neg | neg 1 | nd |
| 113 | Pol3 | F/A | Saly | 153157 | 133145 | 110114 | 131139 | 144154 | 157163 | 12 | neg | neg (1, 2, 7) | neg/nd |
| 114 | Pol3 | F/A | Rita | 153157 | 145165 | 110114 | 131139 | 146 | 163165 | 20 | neg | neg (1, 2, 7) | nd |
| 124 | Pol3 | nd | nd | ns | ns | ns | ns | ns | 157163 | bs | neg | neg (1,2,3,4,7) | nd |
| 125 | Pol3 | F/A | Saly | 153157 | 133145 | 102110 | 131139 | 146156 | 157165 | 11 | nd | nd | nd |
| 132 | Pol3 | M/SA | Remi | nd | nd | nd | nd | nd | nd | nd | neg | neg (1,2,7) | nd |
| 134 | Pol3 | F/A | Sea | 153157 | 145145 | 106110 | 131139 | 144146 | 157165 | 17 | neg | nd | nd |
| 135 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 137 | Pol3 | nd | nd | ns | 133145 | 110114 | 131139 | 146154 | 157165 | 7 | neg | neg (1,2,7) | nd |
| 138 | Pol3 | nd | nd | 153157 | 133165 | ns | ns | ns | 157163 | bs | neg | neg (2,7) | nd |

Table 9.4: Genotype results at 6 loci and SIV results for all samples collected (continued)

| [a]Faecal spl. | Social group | [b]Sex/age | Indiv. name | [c]locus D3s1768 | locus D4s243 | locus D5s1457 | locus D6s501 | locus D7s503 | locus D17s791 | [d]indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 139 | Pol3 | nd | nd | 153157 | 145165 | 110114 | 131139 | 146146 | 163163 | 8 | neg | neg (1, 2, 7) | nd |
| 143 | Pol3 | nd | nd | 153157 | 145145 | 110114 | 131139 | 144146 | 157163 | 9 | neg | neg (1,2,3,4,7) | nd |
| 145 | Pol3 | nd | nd | 153157 | 145165 | 110114 | 135139 | 146146 | 163165 | 10 | neg | neg (1,2,3,4,7) | nd |
| 147 | Pol3 | nd | nd | 153157 | 145145 | 110114 | 131139 | 144146 | 157163 | 9 | nd | nd | nd |
| 154 | Pol3 | F/A | Saly | nd | nd | nd | nd | nd | nd | nd | nd | neg (1, 7) | nd |
| 155 | Pol3 | nd | nd | ns | ns | ns | ns | 138144 | 151157 | bs | neg | neg (1,2,7) | nd |
| 156 | Pol3 | nd | nd | ns | ns | ns | ns | 138144 | 151157 | bs | neg | neg (1,2,7) | nd |
| 157 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | neg (5) | nd |
| 158 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 158b | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 159 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | neg (5) | nd |
| 160 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 161 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 162 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 163 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 164 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 186 | Pol3 | M/SA | Remi | 157161 | 145157 | 106110 | 139143 | 144152 | 163165 | 13 | nd | neg (1,2,3,4,7) | nd |
| 188 | Pol3 | F/A | Macula | 157157 | 145169 | 110110 | 135143 | 138144 | 157163 | 21 | nd | neg (1, 2, 7) | nd |
| 199 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 200 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 201 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 202 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 203 | Pol3 | nd | Sea | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 204 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 216 | Pol1 | nd | nd | 157157 | 137145 | 102110 | 131139 | 152152 | 167167 | 1 | neg | neg (1,2,3,4,7) | nd/pos |
| 217 | Pol1 | nd | nd | 157161 | 141169 | 110114 | 131139 | 152152 | 157163 | 2 | neg | neg (2, 3, 4, 7) | nd/pos |

Table 9.4: Genotype results at 6 loci and SIV results for all samples collected (continued)

| [a]Faecal spl. | Social group | [b]Sex/age | Indiv. name | [c]locus D3s1768 | locus D4s243 | locus D5s1457 | locus D6s501 | locus D7s503 | locus D17s791 | [d]indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 218 | Pol1 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd/pos |
| 219 | Pol1 | nd | nd | 157161 | 145145 | 106110 | 131139 | 138152 | 159165 | 3 | neg | nd | nd/pos |
| 220 | Pol1 | nd | nd | 153157 | 141169 | 110114 | 131139 | 152152 | 157163 | 4 | nd | neg (1,2,5,7) | nd/pos |
| 221 | Pol1 | nd | nd | 157161 | 137145 | 106110 | 131139 | 138152 | 159165 | 5 | nd | neg (1, 2, 7) | nd/pos |
| 222 | Pol1 | nd | nd | 153157 | 137145 | 106110 | 131139 | 152152 | 165167 | 6 | nd | neg (1, 2, 7) | nd/pos |
| 226 | Pol3 | M/A | Soco | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 227 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 234 | Pol3 | M/A | Koly | 157161 | 145157 | 98106 | 139143 | 152152 | 161165 | 14 | neg | neg (1,2,3,4,7) | nd |
| 245 | Pol3 | M/A | Soco | ns | ns | ns | ns | ns | 153167 | bs | nd | neg (1,2,3,4,7) | nd |
| 246 | Pol3 | M/SA | Remi | 157161 | 145157 | 106110 | 139143 | 144152 | 163165 | 13 | neg | neg (1, 2, 3, 4, 5, 7) | nd |
| 255 | Pol3 | F/A | Macula | 157157 | 145169 | 110110 | 135143 | 138144 | 157163 | 21 | nd | nd | nd |
| 265 | Pol3 | M/A | Koly | 157157 | 133157 | 110114 | 131139 | 146152 | 165167 | 15 | neg | neg 1 | nd |
| 277 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 278 | Pol3 | F/A | Macula | 157157 | 145169 | 110110 | 135143 | 138144 | 157163 | 21 | neg | neg (1, 2, 7) | nd |
| 279 | Pol3 | nd | nd | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd |
| 283 | Pol3 | M/A | Soco | 153157 | 137145 | 106110 | 131139 | 144146 | 157165 | 14 | nd | neg 1 | nd |
| 291 | Pol3 | F/A | Macula | nd | nd | nd | nd | nd | nd | nd | neg | nd | nd |
| 303 | Pol3 | M/A | Soco | 153157 | 133145 | 110114 | 131139 | 144154 | 157163 | 12 | neg | nd | nd |
| 13 | Ver3 | F/A | nd | ns | 161173 | 118122 | ns | 134136 | 141151 | 8 | nd | neg (3, 4, 6) | nd |
| 14 | Ver3 | F/A | nd | ns | 161173 | 118122 | ns | 134136 | 141151 | 8 | ni | nd | nd |
| 15 | Ver3 | M/A | Fabius | 153153 | ns | 118154 | 140148 | 132134 | 141151 | 7 | ni | neg (1,3, 4, 6) | neg/nd |
| 48 | Ver1 | M/A | Solo | 153153 | 161177 | 114118 | 144152 | 134138 | 151153 | 1 | ni | neg (3, 4, 6) | nd/pos |
| 50 | Ver1 | F/A | nd | 153157 | 161173 | 110114 | ns | 134136 | 141151 | 3 | ni | neg (3, 4, 6) | nd/pos |
| 55 | Ver1 | M/A | Solo | 153153 | ns | ns | ns | 134138 | ns | bs | ni | neg (3, 4, 6) | nd/pos |
| 56 | Ver3 | F/A | Etranie | 153153 | 161177 | 114118 | 144152 | 134138 | 153153 | 9 | ni | neg (3, 4, 6) | nd |
| 66 | Ver1 | M/A | Solo | 153153 | 161177 | 122154 | 148152 | 134138 | 141151 | 2 | ni | neg (3, 4, 6) | nd/pos |

Table 9.4: Genotype results at 6 loci and SIV results for all samples collected (continued)

| [a]Faecal spl. | Social group | [b]Sex/age | Indiv. name | [c]locus D3s1768 | locus D4s243 | locus D5s1457 | locus D6s501 | locus D7s503 | locus D17s791 | [d]indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 173 | Ver3 | M/A | Fabius | ns | ns | 118126 | 156160 | 138140 | 151181 | 10 | neg | neg (3, 4, 6) | nd |
| 181 | Ver1 | M/A | Solo | nd | nd | nd | nd | nd | nd | nd | ni | nd | nd/pos |
| 272 | Ver2 | M/A | nd | 153153 | 145161 | 118122 | 140148 | 132134 | 151155 | 6 | ni | neg (3, 4, 6) | nd/pos |
| 289 | Ver2 | nd | nd | 153153 | 177 ns | 118126 | 140148 | 132134 | 141151 | 4 | ni | neg (3, 4, 6) | nd/pos |
| 297 | Ver3 | M/A | nd | 153153 | 161177 | 118154 | 140148 | 132134 | 141151 | 7 | ni | neg (3, 4, 6) | nd |
| 298 | Ver2 | nd | nd | 153153 | ns | 110122 | ns | 140144 | ns | 5 | neg | neg (3, 4, 6) | nd |
| 57 | Dia1 | nd | nd | 195203 | 0 | 120124 | 156176 | 142144 | 0 | 1 | neg | neg (1, 2, 5) | neg/nd |
| 61 | Dia1 | nd/A | nd | 211211 | 0 | 116124 | 156164 | 144144 | 0 | 2 | neg | neg (1, 2, 5) | nd |
| 62 | Dia1 | nd | nd | 203203 | 0 | ns | 164172 | 136144 | 0 | 3 | neg | neg (1, 2, 5) | nd |
| 63 | Dia1 | nd | nd | 195195 | 0 | 120124 | 168180 | 142142 | 0 | 4 | neg | neg (1,2,9) | nd |
| 64 | Dia1 | nd/S | nd | 195211 | 0 | 100120 | 148156 | 138140 | 0 | 5 | neg | neg (1, 2, 9, 5) | nd |
| 65 | Dia1 | nd/A | nd | 195207 | 0 | 100124 | 156164 | 140148 | 0 | 6 | neg | neg (1, 2, 9, 5) | nd |
| 71 | Dia1 | nd | nd | 195215 | 0 | 124128 | 164168 | 144148 | 0 | 7 | neg | neg (1, 2, 5) | nd |
| 72 | Dia1 | nd | nd | 195215 | 0 | 124128 | 164168 | 144148 | 0 | 7 | neg | neg (1, 2, 5) | nd |
| 121 | Dia1 | nd | nd | 195195 | 0 | 120128 | 156176 | 146148 | 0 | 8 | neg | neg (1, 2, 5) | nd |
| 136 | Dia1 | nd | nd | 191219 | 0 | 124128 | 152156 | 144146 | 0 | 9 | neg | neg (1, 2, 5) | nd |
| 58 | Dia1 | F/A | nd | 211211 | 0 | 116124 | 156164 | 144144 | 0 | 2 | neg | neg (1, 2, 5) | neg/nd |
| 59 | Dia1 | F/A | nd | 195195 | 0 | 120124 | ns | 142150 | 0 | 10 | neg | neg (1, 2, 5) | nd |
| 70 | Dia1 | F/A | nd | 195195 | 0 | 120128 | 164168 | 148150 | 0 | 11 | neg | neg (1, 2, 5) | nd |
| 34 | Dia2 | nd | nd | 203203 | 0 | ns | 140148 | ns | 0 | bs | neg | neg (1, 2, 5) | nd |
| 76 | Dia2 | nd/S | nd | 195203 | 0 | 112120 | 148168 | 146150 | 0 | 12 | neg | neg (1, 2, 5) | nd |
| 88 | Dia2 | nd/J | nd | 195195 | 0 | 120128 | 164168 | 134144 | 0 | 13 | neg | neg (1, 2, 5) | nd |
| 192 | Dia2 | nd/A | nd | 195203 | 0 | 112120 | 148168 | 146148 | 0 | 14 | neg | neg (1, 2, 5) | nd |
| 210 | Dia2 | nd/A | nd | 207211 | 0 | 120124 | 164172 | 136148 | 0 | 15 | neg | neg (1, 2, 5) | nd |
| 211 | Dia2 | nd | nd | 203203 | 0 | 120128 | 140148 | 146150 | 0 | 16 | neg | nd | nd |
| 36 | Dia2 | M/A | nd | 195215 | 0 | 124128 | 164168 | 144148 | 0 | 7 | neg | neg (1, 2, 5) | nd |

Table 9.4: Genotype results at 6 loci and SIV results for all samples collected (continued)

| [a]Faecal spl. | Social group | [b]Sex/age | Indiv. name | [c]locus D3s1768 | locus D4s243 | locus D5s1457 | locus D6s501 | locus D7s503 | locus D17s791 | [d]indiv. N° | [e]Faecal antibody detection | [f]Faecal vRNA detection | G6pdh/ mtDNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 35 | Dia2 | F/A | nd | 195203 | 0 | 112120 | 148168 | 146150 | 0 | 12 | neg | neg (1, 2, 5) | nd |
| 49 | Dia2 | F/A | nd | 195195 | 0 | 120128 | 164168 | 148150 | 0 | 11 | neg | neg (1, 2, 5) | nd |
| 77 | Dia2 | F/A | nd | 195195 | 0 | 124128 | 148164 | 142146 | 0 | 17 | neg | neg (1, 2, 5) | nd |
| 87 | Dia2 | F/A | nd | 211215 | 0 | 124128 | 152160 | 144150 | 0 | 18 | neg | neg (1, 2, 5) | nd |
| 191 | Dia2 | F/A | nd | 195195 | 0 | 116124 | 148176 | 138150 | 0 | 19 | neg | neg (1, 2, 5) | nd |
| 209 | Dia2 | F/A | nd | 215215 | 0 | 124128 | 148160 | 150150 | 0 | 20 | neg | neg (1, 2, 5) | nd |
| 271 | Dia3 | nd | nd | 203203 | 0 | 116124 | 152156 | 138140 | 0 | 21 | neg | nd | nd |
| 273 | Dia3 | nd | nd | 203203 | 0 | 116124 | 152156 | 138140 | 0 | 21 | neg | nd | nd |
| 288 | Dia3 | nd | nd | 195195 | 0 | 120124 | 156164 | 132144 | 0 | 22 | neg | neg (1, 2, 5) | nd |
| 294 | Dia3 | nd/A | nd | 203215 | 0 | 120124 | 148164 | 138144 | 0 | 23 | neg | neg (1, 2, 5) | nd |
| 287 | Dia3 | F/A | nd | 203215 | 0 | 120124 | 148164 | 138144 | 0 | 23 | neg | neg (1, 2, 5) | nd |
| 51 | Cam1 | nd | nd | 202214 | ns | 128132 | 158170 | 128132 | 0 | 1 | neg | nd | nd/pos |
| 53 | Cam1 | nd/A | nd | 214214 | ns | 128132 | 158170 | 128132 | 0 | 2 | neg | nd | nd/pos |
| 67 | Cam1 | nd/A | nd | 202214 | ns | 128132 | 158170 | 128132 | 0 | 1 | neg | neg (1, 2) | nd/pos |
| 68 | Cam1 | nd/A | nd | 218218 | 150150 | 128132 | 166170 | 130132 | 0 | 3 | neg | nd | nd/pos |
| 73 | Cam1 | F/A | nd | 214218 | 150158 | 128132 | 166170 | 130132 | 0 | 4 | neg | nd | nd/pos |
| 78 | Cam1 | F/A | nd | ns | 150150 | 128132 | 154166 | 132134 | 0 | 5 | neg | nd | nd/pos |
| 85 | Cam1 | F/A | nd | 206218 | 150150 | 132132 | 166166 | 130132 | 0 | 6 | neg | nd | nd/pos |
| 47 | Pet1 | nd | nd | 195203 | ns | 120120 | 147167 | 146150 | 0 | 1 | nd | nd | nd |
| 86 | Pet1 | M/A | nd | 201205 | ns | 124128 | 143167 | 136138 | 0 | 2 | neg | nd | nd/pos |
| 75 | Pet1 | F/A | nd | 205205 | ns | 132132 | 143147 | 138148 | 0 | 3 | neg | neg (1, 2, 9) | nd/pos |
| 74 | Nic1 | nd | nd | 201201 | 180200 | 128132 | 160168 | 148148 | 0 | 1 | neg | neg (1, 2, 9) | nd/pos |
| 165 | Nic1 | F/A | nd | 201205 | ns | 128132 | 140160 | 136148 | ns | 2 | neg | nd | nd/pos |

[a] Number given to the sample collected; [b] M, male ; F, female ; nd, not determined; A, adult; S, sub-adult; J, juvenile; [c] Genotype analysis: ns, not scorable ; nd, not determined; [d]Individual number, number assigned to every sample, which shows the same genetic profile (conservative method). bs, 'bad sample', a sample which DNA quality was not sufficient to provide repeatable and reliable genotype results; [e]ni, not interpretable: absence or too low IgG bands; nd, not determined; neg, negative [f] PCR amplification results: the numbers in parenthesis refer to the PCR strategies and primers used. Neg, no amplification; nd, not determined.

Table 9.5: Socio-ecological characteristics of red, black and white and olive colobus, three species of Cercopithecus monkeys (Diana monkeys, Campbell's monkeys and lesser and greater spot-nosed monkeys), sooty mangabeys and chimpanzees

| | ***P. badius*** | ***C. polykomos*** | ***P. verus*** | ***C. diana*** | ***C. campbelli*** | ***C. petaurista*** | ***C. nictitans*** | ***C. atys*** | ***P.t.verus*** |
|---|---|---|---|---|---|---|---|---|---|
| **Demography** | | | | | | | | | |
| group size[a] | 52.9 | 15.4 | 6.7 | 20.2 | 10.8 | 17.5 | 10.5 | 69.7 | 41 |
| population density (km$^2$) | 123.8 | 35.5 | 17.3 | 48.2 | 24.4 | 29.3 | 2.1 | 11.9 | 2.7 - 4.08 |
| average nbr of adult males[b] | 10.1 | 1.42 | 1.43 | 1 | 1 | 1 | 1 | 9 | 4 |
| longevity (years)[c] | nd | 30.5 | nd | 34.8 | 30.8 | nd | nd | 27 | 32 |
| **Social structure** | | | | | | | | | |
| group type | multi-male | 1-2 males | variable (1-3) | single-male | single-male | single-male | single-male | multi-male | multi-male |
| between groups transfer | F biased | M biased | M, F | M | M | M | M | M | F |
| **Mating system** | polygynandry (promiscuous) | polygyny | polygynandry (promiscuous) | polygyny[d] | polygyny[d] | polygyny[d] | polygyny | mate guarding[e] | female mate choice, consortship[f] |
| **Reproduction** | | | | | | | | | |
| seasonality | yes | no | yes | yes | yes | yes | yes | yes | no |
| age at sexual maturity: | | | | | | | | | |
| males | nd | nd | nd | nd | 4.5 | nd | 5-6 | nd | 15 |
| females (first reproduction) | 4.1 | 8.5 | nd | 5.4 | 4 | nd | 5 | 4.7 | 13.7 |
| interbirth interval (years) | ~2 | ~1 | 1.6 | ~1 | ~1 | nd | ~2 | ~2 | 5.75 |
| average nbr of newborns/female (year) | 0.42 | 0.59 | 0.61 | 0.62 | 0.63 | 0.52 | 0.5 | 0.4 | 0.23 |
| **Ecology** | | | | | | | | | |
| range (km$^2$) | 0.58 | 0.78 | 0.56 | 0.63 | 0.6 | 0.69 | 0.96 | 4.92 | 16-27 |
| diet: | | | | | | | | | |
| plants | LV>FR | FR=LV | LV>FR | FR>INV>LV | FR>INV>LV | FR>INV>LV | FR>INV>LV | FR>INV>LV | FR>MM/INV/LV |
| invertebrates | no | no | no | yes | yes | yes | yes | yes | yes |
| mammals | no | no | no | no | no | no | no | no | yes |
| **Polyspecific associations** | frequent (*C.diana, P.verus, C.petaurista, C.campbelli* ) | rare (*C.diana*) | very frequent (*C.diana*) | very frequent (*C.petaurista, C.campbelli, P.verus, P.badius, C.atys*) | quite frequent (*C.petaurista, C.diana, P.verus*) | quite frequent (*C.Campbelli, C.diana, P.verus*) | nd | quite frequent (*C.diana*) | no |

[a]data from the period 1992-1995; [b][32, 239]; [c][32, 240]; [d]sexual monopoly of the resident male: allowance of non-residents during breeding season; [e]invasion of non-resident males during the breeding season; [f]high intra-male competition for fertile females; [g]calculated on the basis of the birth rate per year [310]; FR:Fruits; LV: leaves; INV: invertebrates; MM: mammals; data of *C.diana*, *C.campbelli*, *C.petaurista* and *C.nictitans* are from [46, 74, 414, 426]

# 10. Simian immunodeficiency virus analysis of 5 blood samples from the chimpanzee subspecies *Pan troglodytes verus*, from Taï National Park, Côte d'Ivoire

Sabrina Locatelli[1], Florian Liegeois[1], Pierre Formenty[2], Eric Delaporte[1], Martine Peeters[1]

[1]UMR 145, Institut de Recherche pour le Développement (IRD), and University of Montpellier 1, Montpellier, France

[2]Ebola Taï Forest Project, Abidjan, Côte d'Ivoire; World Health Organisation (WHO), Department of Communicable Diseases, Surveillance and Response (CDS/CSR), Geneva, Switzerland

**This is an article in preparation**

## 10.1 Abstract

It is well established today that SIVcpz from the chimpanzee *Pan troglodytes troglodytes* is one of the precursor of HIV-1, a virus which has already infected more than 60 million people in the world. Four subspecies of chimpanzee are currently recognised, but only *P.t.troglodytes* from West-central Africa and *P.t.schweinfurthii* from East Africa have been found to be infected with SIVcpz. SIVcpz is a mosaic virus issued from the recombination of SIVs infecting monkeys which chimpanzees prey upon. It is known that also western chimpanzees (*Pan troglodytes verus*) hunt other non-human primates, in particular colobus monkeys. We know at least for western red colobus that they are infected with SIVwrc at a relatively high prevalence. Therefore, our aim was to determine whether West African chimpanzees would harbour SIV-monkey like viruses. We analysed 5 blood samples from *Pan troglodytes verus* collected from carcasses found in the Taï Forest in the late nineties. Three out of five samples reacted weakly with the antigen for the transmembrane envelope glycoproteine gp41, the exterior envelope glycoproteine sgp105, the core protein p24 or the matrix protein p17. However, despite the positive INNO-LIA assay results, we could not isolate and further characterise any SIV with PCR techniques.

**Keywords:** *Pan troglodytes verus*, western chimpanzee, Taï Forest, SIVcpz, SIV

## 10.2 Introduction

Numerous African primates are infected with simian immunodeficiency viruses (SIVs), and it is now well established that SIVs infecting chimpanzees (*Pan troglodytes troglodytes*) and western gorillas (*Gorilla gorilla gorilla*) in West-central Africa are the progenitors of human immunodeficiency virus type 1 (HIV-1) [122, 183, 382, 386].

Chimpanzees from West-central Africa, acquired their SIV infection through hunting other NHP species [242, 338]. In fact, the SIVcpz genome is the result of cross-species transmissions and recombination, with its 5' end being closest to that of SIVrcm from red capped mangabeys and the 3' end most closely related to those of the SIVgsn/SIVmus/SIVmon lineage from greater spot nosed, moustached and mona monkeys [14, 23, 79, 323]. Chimpanzees can be divided into four distinct subspecies according to mitochondrial DNA (mtDNA) sequences [116, 134].The different subspecies are also geographically separated: *Pan troglodytes verus* is restricted to West Africa from southern Senegal to Ivory Coast, *Pan troglodytes troglodytes* is present across West-central Africa from southern Cameroon to the Oubangui River in Congo, *Pan troglodytes schweinfurthii* lives in East-central Africa including eastern Congo (DRC), Uganda, Ruanda, Burundi and Tanzania; and finally, *Pan troglodytes vellerosus* are genetically distinct chimpanzees restricted to a small geographic region between the Cross-River in Nigeria and the Sanaga River in Cameroon [139]. Only *P. t. troglodytes* and *P. t. schweinfurthii* are known to harbour SIVcpz, and their viruses form divergent subspecies-specific phylogenetic lineages (SIVcpz*Ptt* and SIVcpz*Pts*) [338]. All HIV-1 strains fall within the SIVcpz*Ptt* lineage from West-central Africa and no human counterpart has yet been identified for SIVcpz*Pts* in chimpanzees from East-central Africa [183, 320, 321, 338, 384].

Despite extensive testing for HIV cross-reactive antibody detection, naturally occurring lentiviruses have not been detected in West African chimpanzees (*P.t.verus*). More than 1400 samples were tested, 264 were African born animals and presumably captured when they were infants. The subspecies has been determined in 161 individuals and 143 were *Pan troglodytes verus* [303, 362]. Cameroon is also home to the *Pan troglodytes vellerosus* chimpanzee subspecies, but no case of SIVcpz infection has thus far been identified in this subspecies, although only about a hundred samples have been so far analysed [183, 257, 384].

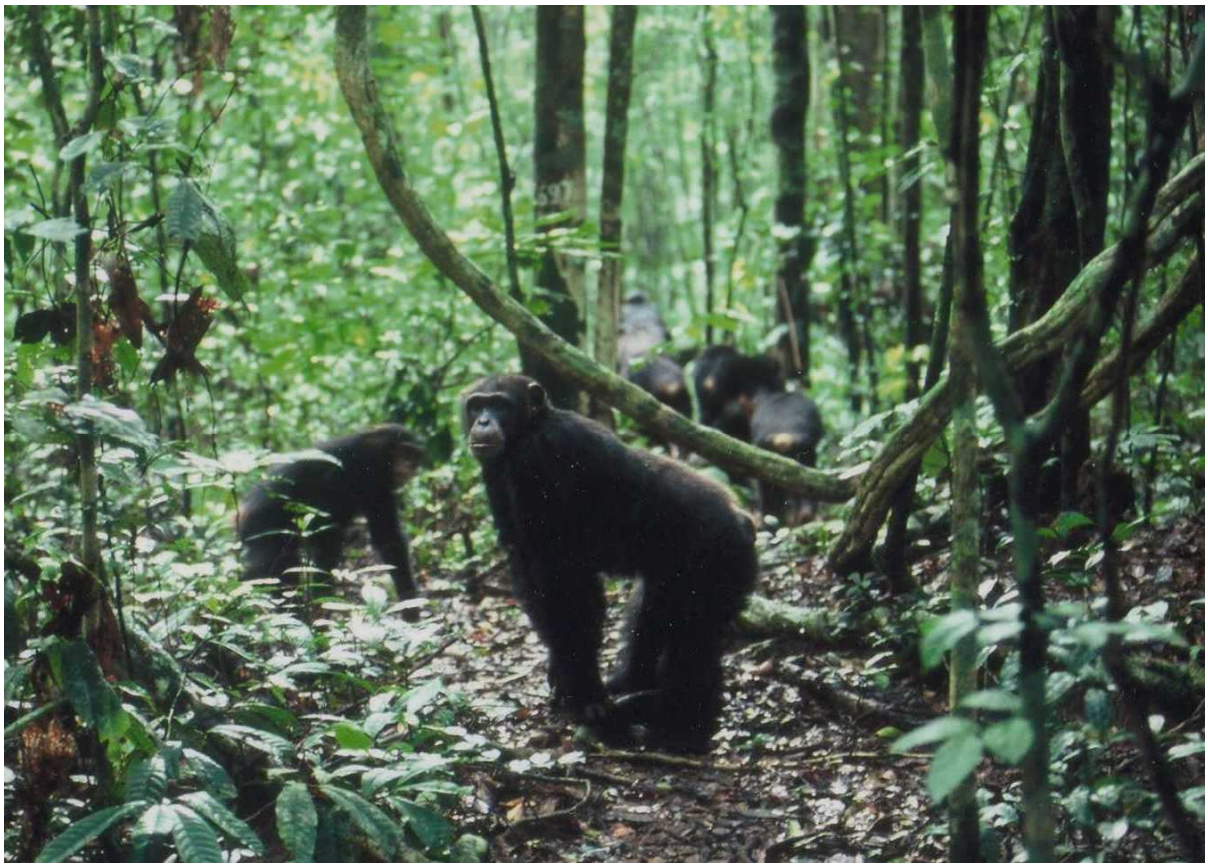
One of the explanations currently given for the lack of infection in two subspecies of chimpanzees is that the introduction of SIV in this ape species probably occurred after the geographic separation and divergence of *P.t.troglodytes* and *P.t.schweinfurthii* from *P.t.verus* and *P.t.vellerosus*, but before the subspeciation of *Ptt* and *Pts* [338]. Another explanation is the current lack of specific molecular tools to detect a possibly divergent SIV. We know that the western subspecies of chimpanzee also preys on monkeys species. The chimpanzees from the Taï Forest share their habitat with 8 diurnal monkey species, 3 colobus genera, 4 *Cercopithecus* species and the sooty mangabeys, which SIVsmm has been identified as the progenitor of HIV-2 [66, 322]. These chimpanzees are strongly specialised in hunting red colobus monkeys, which are infected with SIVwrc [77, 220]. Although less frequently, the other NHP species were also observed to be preyed upon by chimpanzees [32]. We therefore wanted to investigate whether the western chimpanzee could harbour a mosaic SIV issued from cross-species transmissions and recombination of SIVs harboured by its prey, in analogy with the known SIVcpz.

In this study, we report the serological and molecular results on a few chimpanzee blood samples collected in the Taï Forest during an Ebola survey conducted by the World Health Organisation in the late nineties. Three out of five samples reacted positively when tested with the INNO-LIA cross-reactive HIV-1/HIV-2 test, but could not be confirmed by Polymerase Chain Reaction (PCR).

## 10.3 Materials and Methods

### Study site and samples collection

The present study was carried out on 5 blood samples collected from chimpanzees' carcasses in Taï National Park, Côte d'Ivoire, during a WHO survey on Ebola at the end of the nineties. These chimpanzees belonged to the habituated social groups studied by the research team of Christophe Boesch. Further details on the study site and the chimpanzees are given in "The Chimpanzees of the Taï Forest: Behavioural ecology and evolution" [32].



Picture 10.1: Chimpanzees from Taï National Park, Côte d'Ivoire (S.Locatelli)

### Detection of HIV cross-reactive antibodies in faecal samples

These 5 blood samples were previously tested for HIV cross-reactive antibodies by the INNO-LIA HIV confirmation test (Innogenetics, Ghent, Belgium) as previously described [287]. This test includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip. Samples were scored as INNO-LIA positive when they react with at least one HIV antigen and have a band intensity equal to or greater than the assay cut-off (+/-) lane; samples

that react less strongly but still visibly with two or more HIV antigens were classified as indeterminate; and samples reacting with no bands or only one band with less than +/- intensity were classified as negative.

**Amplification of SIV from blood/tissue DNA**

Total DNA was isolated from whole blood samples using the QIAamp blood kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. We performed PCRs of integrated genomic DNA using species specific as well as universal primers: the primers' sequences, annealing temperatures, amplicon sizes, targeted region of the SIV genome and references are listed in Table 10.1. PCR amplifications were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Manheim, Germany) according to the manufacturer's instructions. Each amplification reaction included a manual hot-start at 94℃ followed by 35 cycles with a denaturation step at 94℃ for 20sec, an anne aling temperature set according to the primers' melting temperatures and a variable extension time depending on the size of the expected fragment (1mn/kb). PCR products were purified (Q-Biogene, Illkirch, France) and directly sequenced using the inner primers on an ABI 3130xl Genetic Analyser (Applied Biosystem, Courtaboeuf, France). We then checked and assembled the sequences using the software package Lasergene (DNASTAR Inc. Madison, USA).

## 10.4 Results

**Detection of HIV cross-reactive antibodies**

As previously tested, 3 samples out of 5 reacted positively to some of the HIV antigens: sample 1099 reacted with the antigen for the transmembrane envelope glycoproteine gp41 and weakly to the exterior envelope glycoproteine sgp105; sample 1199 reacted with the antigen for the gp41 and the core protein p24 and sample 0600 reacted with the antigen for the gp41 and weakly with the matrix protein p17 (Figure 10.1).

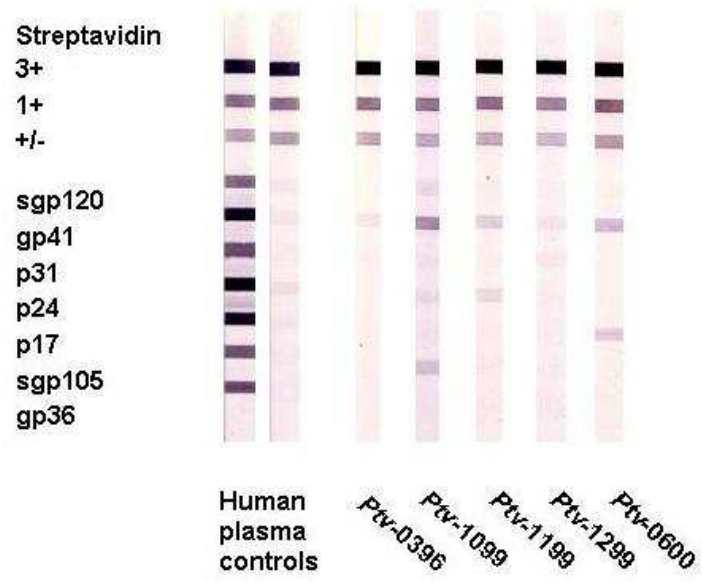



Figure 10.1: Detection of HIV cross-reactive antibodies in blood samples from wild-living chimpanzees. Samples were tested with the INNO-LIA HIVI/II score confirmation test. Samples are numbered according to the subspecies and the date of collection. Molecular weights of HIV-1 proteins are indicated. A positive HIV-1 infected human and a non infected human used as a negative control are shown.

**Detection of SIV infection in chimpanzees from the Taï Forest by amplification of viral DNA in blood and tissue samples**

We could confirm a good quality of the DNA extracted by G6PDH amplification in four out of five chimpanzee samples. We tested nonetheless all five tissue samples with universal primers, targeting the *pol* region of the SIV genome, with specific primers amplifying *pol* and *env* regions in the subspecies of chimpanzee *Pan troglodytes troglodytes* from Cameroon [183, 385], with SIVwrc primers amplifying *pol*, *env* and *gag* fragments in red colobus [219], with consensus primers designed from the alignment of SIVwrc/SIVolc/SIVcol sequences and finally, with primers amplifying *gag* and *env* regions of SIVsmm isolated from sooty mangabeys. We wanted to investigate whether the subspecies *P.t.verus* could harbour a SIV which genome would be, at least in part, similar to that of the red colobus, a species highly preyed upon by chimpanzees or similar to other monkey species sharing the same territory. In spite of the positive results obtained in three chimpanzees samples

tested with the INNO-LIA HIV confirmation assay, all the PCR tests we performed were negative (Table 10.1).

Table 10.1: Primers tested and INNO-LIA and PCR results on 5 samples of *Pan troglodytes verus*

| **PCR strategies tested/Individual tested** | ***Ptv*-0396** | ***Ptv*-1099** | ***Ptv*-1199** | ***Ptv*-1299** | ***Ptv*-0600** |
|---|---|---|---|---|---|
| INNO-LIA test | neg | **pos** | **pos** | neg | **pos** |
| DR1-DR2/DR4-DR5 (1) | neg | neg | neg | neg | neg |
| polOR-polis4/polis2uni2 (2) | neg | neg | neg | neg | neg |
| wrcpol (3) | neg | neg | neg | neg | neg |
| wrcenv (4) | neg | neg | neg | neg | neg |
| wrcgag (5) | neg | neg | neg | neg | neg |
| olcpol (6) | nd | neg | neg | nd | neg |
| bwcpol (8) | nd | neg | neg | nd | neg |
| polis4-SIVenvR/lhoenv (10) | neg | neg | neg | neg | neg |
| polis4/PolOr polF2/polR2 (11) | neg | neg | neg | neg | neg |
| gp40F1-gp41R1 gp46F2/gp48R2 (12) | neg | neg | neg | neg | neg |
| SPBSwrcgagR1/SPBSwrcgag (13) | neg | neg | neg | neg | neg |
| smmgag (14) | neg | neg | neg | neg | neg |
| smmenv (gp41) (15) | neg | neg | neg | neg | neg |
| g6pdh (16) | neg | **pos** | **pos** | **pos** | **pos** |

## 10.5 Discussion

We observed a positive signal on the INNO-LIA strips in 3 out of 5 samples from the chimpanzee subspecies *P.t.verus.* However, despite the PCR analysis using a wide variety of universal and species specific primers, we did not obtain any positive result confirming the serological tests. The possibility of failing to amplify serologically positive samples has to be considered together with false positive serological results due to unspecific antibody cross-reactivity, as observed in studies conducted on *Pan troglodytes troglodytes* faecal samples from Cameroon [183, 385]. Although it is today demonstrated that chimpanzees of the *P.t.t* subspecies are the reservoir for SIVcpz [183, 323, 385], with a prevalence of infection of up to 35%, available data fail to demonstrate that SIVcpz coevolved with its host [303, 323, 362]. Therefore, one hypothesis for the lack of evidence of SIVcpz infection in two of the four chimpanzee subspecies is that chimpanzees acquired this recombinant virus, or its progenitors, by cross-species transmission some time after the split of *P. t. verus* and *P. t. vellerosus* from the other subspecies, but possibly before the divergence of

*P. t. schweinfurthii* from *P. t. troglodytes* about 1.5 million years ago [338]. Alternatively, it is this prolonged isolation of the chimpanzee subspecies which is responsible for a yet undetected, widely different SIV subtype. However, five individuals sampled out of a community which could be composed of more than 50 individuals is not a sufficient number to draw any supported conclusion. More samples should be collected, but the logistics are sometimes daunting, giving the political and social instabilities of the countries in which the chimpanzee populations are located. Moreover, chimpanzee populations as well as other primate populations are disappearing at an alarming rate due to hunting and deforestation [189, 237, 238, 310]. Chimpanzees from the subspecies *Pan troglodytes verus* should be kept under investigation, since it is know that they heavily prey on colobus monkeys and in particular on red colobus, a species with a relatively high prevalence of SIVwrc infection. In addition, the data obtained on simian T-cell leukaemia viruses type-1 isolated from chimpanzees in the Taï Forest emphasises the possibility of interspecies viral transmission through predatory relationships [208, 209].

Moreover, also the human population is exposed to the risk of getting in contact with a virus by handling and consuming the flesh of potentially infected animals. In Taï Forest, out of 3500 animals confiscated form poachers between 1993 and 1997, chimpanzees represented only 0.09 % [53]. However, this is certainly an underestimation: given the heavy weight of the carcasses, chimpanzee meat is cut and smoked before transport and often consumed in villages instead of being transported to big markets.

In conclusion, an extensive sampling of wild-living *Pan troglodytes verus* should be carried out across West Africa before confirming any suppositions on the absence of SIV in this chimpanzee subspecies.

## 10.6 Acknowledgments

We kindly thank Pierre Formenty for the provision of the chimpanzee samples collected in Taï National Park. This study was financially supported by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de Recherches pour le SIDA (ANRS).

Table 10.2: Details on primers tested

| Primers tested | Sequences | TM(%GC) (℃) | Estimated amplicon size | Region targeted | Reference |
|---|---|---|---|---|---|
| DR1-DR2/DR4-DR5 (1) | DR1 (5'-TRCAYACAGGRGCWGAYGA-3') | 58 | 800 | *pol* | [67] |
| | DR2 (5'-AIADRTCATCCATRTAYTG -3') | 42.7 | | | |
| | DR4 (5'-GGIATWCCICAYCCDGCAGG-3') | 60 | 200 | | |
| | DR5 (5'-GGIGAYCCYTTCCAYCCYTGHGG -3') | 64 | | | |
| polOR-polis4/ polis2uni2 (2) | polOR(5'-ACBACYGCNCCTTCHCCTTTC -3') | 53.1 | 800 | *pol* | [78] |
| | polis4(5'-CCAGCNCACAAAGGNATAGGAGG-3') | 55.2 | | | |
| | polis2(5'-TGGCARATRGAYTGYACNCAYNTRGAA-3') | 54.7 | 650 | | |
| | uni2(5'-CCCCTATTCCTCCCCTTCTTTTAAAA -3') | 53.3 | | | |
| wrcpol (3) | wrcpolF1 ( 5'-TAGGGACAGAAAGTATAGTAATHTGG-3') | 50.9 | 1100 | *pol* | [220] |
| | wrcpolR1 (5'-GCCATWGCYAA TGCTGTTTC-3') | 49.7 | | | |
| | wrcpolF2 (5'AGAGACAGTAAGGAAGGGAAAGCAGG-3') | 54.4 | 650 | | |
| | wrcpolR2 (5'-GTTCWATTCCTAACCACCAGCADA-3') | 51.4 | | | |
| wrcenv (4) | wrcenvF1 (5'-TGGC AGTGGGACAAAAATATAAAC-3') | 50 | 750 | *env* | [220] |
| | wrcenvR1 (5'-CTGGCAGTCCCTCTTCCA AGTT GT-3') | 55.3 | | | |
| | wrcenvF2 (5'TGATAGGGMTGGCTCCTGGTGATG3') | 56.6 | 550 | | |
| | wrcenvR2 (5'-AATCCCCATTTYAACCAGTTCCA-3') | 51.1 | | | |
| wrcgag (5) | wrcgagF1 (5'-ATDGAGGATAGAGGNTTTGGAGC-3') | 50.2 | 600 | *gag* | [218] |
| | wrcgagR1 (5'-GCCCTCCTACTCCTTGACATGC-3') | 53.4 | | | |
| | wrcgagF2 (5'-CCAACAGGGTCAGATATAGCAG-3') | 49.7 | 250 | | |
| | wrcgagR2 (5'-ACTTCTGGGGCTCCTTGTTCTGCTC-3') | 55.9 | | | |
| olcpol (6) | olcpolF1(5-TAGATACAGGRGCAGATGAYACAGTAAT-3') | 53.8 | 700 | *pol* | unpublished |
| | olcpolR1 (5'TCCAYCCYTGAGGHARYACATTATA-3') | 47.7 | | | |
| | olcpolF2 (5'-CTAGAATWATWGGRGGRATAGGRGG-3') | 52.6 | 300 | | |
| | olcpolR2 (5'-ATYTTWCCTTCTKCTTCYARTCTRTCACA-3') | 50.8 | | | |
| bwcpol (8) | bwcpolF1 (5'-TAGATACAGGAGCAGATGATACAGT-3') | 49.3 | 1000 | *pol* | unpublished |
| | bwcpolR1 (5'-ATTDCCYCCTATCCCTTTATGWGC-3') | 52.7 | | | |
| | bwcpolF2 (5'-AGAYTRGAAGCAGARGGAAAAAT-3') | 44.8 | 600 | | |
| | bwcpolR2 (5'-TCCYACCAATTTYTGTAYATCATTTACTGT-3') | 51.1 | | | |

Table 10.2: Details on primers tested (continued)

| Primers tested | Sequences | TM(%GC) (℃) | Estimated amplicon size | Region targeted | Reference |
|---|---|---|---|---|---|
| polis4-SIVenvR/lhoenv (10) | polis4(5'-CCAGCNCACAAAGGNATAGGAGG-3') | 55.2 | 3600 | *env* | unpublished |
| | SIVENVR (5'-YTBYTGCTGCTGCAMTATCCC-3') | 51.6 | | | |
| | lhoenvF2 (5'- AATCAGATAGTNYAGCAAGCATGG-3') | 48.9 | 600 | | |
| | lhoenvR2 (5'-CCATTAAAKCCAAAGAAGCTACT-3') | 48.3 | | | |
| polis4/PolOr polF2/polR2 (11) | polOR(5'-ACBACYGCNCCTTCHCCTTTC -3') | 53.1 | 800 | *pol* | [320] [386] |
| | polis4(5'-CCAGCNCACAAAGGNATAGGAGG-3') | 55.2 | | | |
| | POLF2 (GGAAGTGGATACTTAGAAGCAGAAGT-3') | 53.3 | 330 | | |
| | POLR2 (5'-CCCAATCCCCCCTTTTCTTTTAAAATT-3') | 52.3 | | | |
| gp40F1-gp41R1 gp46F2/gp48R2 (12) | gp40F1(5'-TCTTAGGAGCAGCAGGAAGCACTATGGG-3') | 64.4 | 850 | *env* | [420] |
| | gp41R1(5'- AACGACAAAGGTGAGTATCCCTGCCTAA-3') | 62 | | | |
| | gp46F2(5'-ACAATTATTGTCTGGTATAGTGCAACAGCA-3') | 59.4 | 445 | | |
| | gp48R2(5'-TCCTACTATCATTATGAATATTTTTATATA-3') | 46.9 | | | |
| SPBS-wrcgagR1/ SPBS-wrcgagR2 (13) | SPBS (5'-GGCGCCCGAACAGGGACTTG-3') | 68 | 1500 | LTR-*gag* | unpublished |
| smmgag (14) | smmgagF1 (5'-TGGGAGATGGGCGCGAGAAACTCCGTC-3') | 60.7 | 1000 | *gag* | [217] |
| | smmgagR1 (5'-ATCAGCAGTGTCTGTGTCATCCAATT-3') | 51.3 | | | |
| | smmgagF2 (5'-AGGGAAAAAAGCAGATGAATTAGAA--3') | 46 | 800 | | |
| | smmgagR2 (5'-GCTCTTGTAGAAYCTATCTACATA-3') | 45.4 | | | |
| smmenv (gp41) (15) | smmenvF1 (5'-GCTACGGCAGGTTCTGCAATGGG-3') | 55.5 | 650 | *env* | [217] |
| | smmenvR1 (5'-CTGGTCCTTGCGGATATGGATCTG-3') | 54 | | | |
| | smmenvF2 (5'-GCTGTCCGCTCAGTCCCGGACTTT-3') | 57.4 | 490 | | |
| | smmenvR2 (5'-GGAGGAGAACACTGGCCTATA-3') | 49.2 | | | |
| g6pdh (16) | g6pdhF1 (5'-CATTACCAGCTCCATGACCAGGAC-3') | 55.3 | 1500 | *g6pdh* gene | [79] |
| | g6pdhR1 (5'-GTGTTCCCAGGTGACCCTCTGGC-3') | 57.9 | | | |
| 12S-L1091/12S-H1478 (17) | 12S-L1091 (5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') | 96 | 390 | 12srRNA gene mtDNA | [383] |

Y=C/T, W=A/T, R=A/G, H=A/C/T, B=C/G/T, S=G/C, K=G/T, D=A/G/T, N=A/C/T/G Y=C/T, M=A/C, K=G

# 11. General discussion

HIV/AIDS is a human infectious disease of zoonotic origin and since its emergence in the late 1970's it has become one of the deadliest pandemics in history. The simian source of HIV is acknowledged today, as both SIVsm/HIV-2 and SIVcpz/HIV-1 fulfil the criteria of (i) similarities in viral genome organisation; (ii) close phylogenetic relationships between SIV and HIV; (iii) SIV prevalence in the natural host; (iv) geographic coincidence and (v) plausible routes of transmission. Since the discovery of the first simian relative of HIV in macaques in 1984, primate lentiviruses have been characterised in at least 40 primate species in Africa. However, more than 20 African species still need to be investigated. Characterisation of these viruses revealed a high genetic diversity and varying levels of prevalence in primate populations (for a review see Van de Woude and Apetrei, 2006). Only 11 cross-species transmission events have been documented and only four of these cross-over events could spread in the human population. They are HIV-1 group M, the major group of viruses of the pandemic, group O, which is responsible for about 5% of cases in Cameroon and surrounding countries and groups A and B of HIV-2, which are the epidemic forms of HIV-2. The other viruses are extremely rare in humans. Despite the frequent occurrence of exposure to SIV-infected monkeys in Central and West Africa, especially by hunting and handling of bushmeat and by keeping NHPs as pets, these findings indicate that cross-species transmission of SIV is not in itself sufficient for the spread into the human population and the generation of an epidemic. Humans have been exposed to SIV infected bushmeat for thousands of years, but AIDS only emerged in the 20$^{th}$ century. Social changes (worldwide urbanisation, intravenous drug abuse, and change in sexual practices), demographic changes (increased and accelerated human mobility and a growing number of refugees), iatrogenic factors (increasing injections and the use of non-sterile needles and syringes) as well as unsafe transfusion practices could account at least in part for the spread of HIV/AIDS. It is clear that we need to learn more about what launches animal viruses into epidemics or pandemics and why most animal viruses fail to successfully establish a sustained human-to human transmission. In order to understand how this disease was able to spread so rapidly, we need to go back to the origin of HIV. It is equally important to better characterise the plethora of SIV

infecting African NHPs, especially considering the alarming increase in the rate of bushmeat hunting in remote tropical forests. Improving our knowledge on the diversity and prevalence of viral strains to which our species is exposed could perhaps allow us to detect and control a local human emergence before it has a chance to spread globally.

I attempted in this thesis to characterise further new SIVs and to determine the prevalence of infection in wild-living NHP populations. I focused in particular on the red colobus, a species heavily hunted by the human population.

In chapter 5, I presented the screening results of 16 microsatellite loci in 3 species of *Colobus* and 4 species of *Cercopithecus* monkeys. The inferred genotypes were utilised as supplementary information for individual discrimination of the faecal samples collected. The deduced number of individuals was considered in the studies of SIV prevalence effectuated on two social groups of western red colobus (chapter 6) and on several other habituated groups of *Colobus* and *Cercopithecus* monkeys (chapter 9). To get a better understanding of the prevalence of SIV infection and the likelihood of cross-species transmissions, I also looked into the behavioural and ecological characteristics of these species as well as their vulnerability to hunting pressure. In chapter 7, the complete genome of an SIV infecting a subspecies of western red colobus (*P. badius temminckii*) from the Gambia was characterised and the possible evolutionary origin of this virus was explored. In Chapter 8, I briefly discussed the recent findings where other complete SIV genomes from the *P. badius badius* subspecies (SIVwrc) and from the *Procolobus verus* species (SIVolc) have been characterised. Chapter 10 reports the results on the screening of *Colobus*-like and *Cercopithecus*-like SIVs in a few western chimpanzee blood samples collected in a previous study.

The key findings of this study are (i) a high prevalence of SIV infection in habituated wild-living red colobus groups and the implication that this result has on the likelihood of cross-species transmission of this virus into the human population and (ii) the determination of SIVwrc and SIVolc as species specific SIV lineages although distantly related to the SIVlho lineage.

The results of the work presented here are of significance for (i) the completion of a genotype database for additional suitable human markers amplifying microsatellite loci in *Colobus* and *Cercopithecus* monkeys; (ii) the host genotype traceability of a selected number of NHP individuals, whose identification is also supported by visual recognition; (iii) the viral genotype traceability of a selected number of identified individuals moving between neighbouring groups; (iv) the estimation of SIV prevalence in wild-living NHP populations considering their socio-ecological characteristics; (v) the advancement in understanding of the driving forces in the evolutionary history of this virus.

One of the strengths of this work has been the combination of different scientific disciplines to attempt the study of zoonotic transmissions by approaching the virus, its host and its host's social structure. Additionally, this study is one of the few which has been conducted on wild-living populations. Consequently, our work is affected by several methodological shortcomings that have already been described in the previous chapters, but that will be discussed more in depth in this section of the thesis.

The general discussion will focus on the following topics:

- The advantages and pitfalls of a non-invasive research approach, with regards to host DNA genotyping, viral RNA isolation from cell debris and antibodies extracted from faecal samples
- The importance of linking SIV epidemiology to the behavioural-ecological structure of the non-human primate societies
- The failure to detect SIV in the West African chimpanzee
- The SIV phylogenetic characteristics of SIVwrc and SIVolc with respect to virus-host co-evolution versus virus cross-species transmission
- The exposure to SIV and other pathogens by hunting and handling bushmeat as a conservation and public health issue
- The risks of emergence of a novel HIV type

## 11.1 Advantages and pitfalls of a non-invasive research approach

I had the chance to collect data for this thesis from NHPs which have been habituated to the presence of human observers. However, this does not necessarily guarantee proximity and easy observation. Especially for arboreal primates, observation remains challenging and opportunities to collect faecal samples can be very rare. Factors such as the monkeys' high position in the canopy, poor visibility due to foliage density, rainy weather, flooded ground or simultaneous 'group defecation' are all obstacles to successful faecal sample collection. The decision was taken not to attempt for large scale invasive sampling (i.e. darting) which could have jeopardised the habituation efforts accomplished over many years and may also have potentially affected the lives of the individuals sampled. Working with faecal samples can be very demanding indeed, but has the advantage of avoiding stress and contact with highly endangered populations.

Below, I will discuss the advantages and limitations encountered using non-invasive methods of DNA and RNA extraction and antibody isolation from faecal samples.

**DNA extraction – Host genotyping**

Individual discrimination can be determined by genotyping several microsatellite loci. Most of the NHP species investigated in this study have never been screened for microsatellites before and no species-specific primers were available. The selection of loci is a lengthy process that requires either the painstaking isolation of candidate loci in the species of interest by screening genomic libraries with appropriate probes or the use of loci originally derived from a closely related species. I opted for the use of primers for microsatellites loci which have originally been isolated from human DNA and that were found to yield amplification products in several primate species [72, 136]. Cross-amplification between divergent taxonomic species, however, has its limits, because at the sequence level, numerous substitution and/or insertion/deletion events occur, possibly modifying the molecular structure of regions flanking the microsatellites [68]. This can lead to unsuccessful microsatellite amplification or to non-specific amplification artefacts, known as 'false alleles'. If a false allele is generated at a homozygous locus, then an individual would

be incorrectly recorded as a heterozygote. This risk can be reduced by selecting trinucleotide or tetranucleotide microsatellites rather than dinucleotides.

Generally, the limits encountered when analysing faecal samples for host genotyping result from either low DNA quantity or low DNA quality. The total DNA extracted from each sample is derived not only from the source individual, but may also contain DNA from dietary components, parasites and commensal bacteria. Moreover, most DNA will be degraded into short fragments, therefore amplifying long DNA sequences may be difficult. I overcame these two problems by (i) genotyping the selected loci in a subset of high quality DNA samples (blood extractions obtained from other studies), in order to have a reliable reference, and (ii) by choosing PCR primers that amplify short DNA fragments (<200-300 base pairs in humans).

Avoiding genotyping errors is very important, because they can strongly affect individual identification studies by erroneously increasing the number of genotypes that are observed in a population sample, or, on the contrary, by ascribing faecal samples from different individuals to a single individual. Allelic dropout produces false homozygotes and can be explained by sampling stochasticity, namely when pipetting template DNA in a very diluted DNA extract, sometimes only one of the two alleles is pipetted, amplified and detected. It is therefore imperative to repeat experiments using a multi-tube approach [253, 363] or/and compare results with DNA samples obtained from blood or tissue. In this study, I also had the chance to check the origin of faecal samples by comparing it, when possible, with the identification of known individuals by visual observation.

In conclusion, despite the limitations of this approach, I could select a series of microsatellite markers sufficient to reliably discriminate the faecal samples collected. In the future, additional highly polymorphic microsatellites should be screened to answer questions about sex composition, individual relationship, dispersal and comparative levels of genetic diversity among social groups.

**SIV Antibody extraction**

Many SIVs have been discovered because blood samples from their primate hosts had antibodies that cross-reacted with HIV-1 and/or HIV-2 antigens [21, 27, 28, 76-78, 87, 99, 122, 127, 275, 277, 287, 350, 364]. In this thesis and for the first time, we provided results on cross-reactive HIV antibody tests performed on faecal samples from NHP species other than great apes. As described in chapter 6 and 9,

Line immuno assays antibody tests were used to screen *Colobus* and *Cercopithecus* faecal samples.

The results showed that we could not be confident with most of the serological results obtained in the *Colobus* species. In contrast, reliable serological results for the *Cercopithecus* faecal samples were obtained. Therefore, we speculated that the chemical properties of the food ingested by colobines, particularly rich in tannins, their enlarged salivary glands influencing the digestive processes and the presence of pancreatic ribonucleases [326] may be involved in antibody capture and/or faster degradation of the nutrients ingested and of the faecal matter. The colobines' stomach is large and multi-chambered, and the forestomach (presaccus plus saccus) supports a bacterial microflora with cellulose digesting abilities [182]; in contrast, the cercopithecines have simple stomachs and diet richer in fruits and insects [88].

These results demonstrate that antibody detection tests cannot be used to test faecal samples from all the studied primate species. Moreover, primate lentiviruses are extremely diverse; therefore it is unlikely that an assay based on a single HIV or SIV strain could be sufficiently cross-reactive to reliably detect antibodies directed against other strains.

Alternative methods using synthetic linear or multiple antigenic peptides (MAP's) encompassing the V3-loop region and/or the immunodominant gp41 ectodomain of the SIV envelope glycoprotein have been tested as antibody capture antigens to be used in Enzyme Linked ImmunoSorbent Assays (ELISAs) [233, 254, 346]. gp41 proteins are used to detect a wide variety of SIV strains from different lineages, but because this approach is highly sensitive, the observed cross-reactivity may not be species-specific and may produce false positive results. Antibody reaction against synthetic V3 loop peptides is more specific. With this approach, numerous strain-specific antibodies have been discovered in primates [2]. However, peptide design has not targeted yet all SIV strains known to date and their utility in detecting antibodies extracted from faecal samples of diverse SIV infected wild primate populations remains to be tested.

**Viral RNA extraction from faecal samples and SIV sequence fragments amplification**

In this thesis the feasibility and utility of non-invasive approaches to molecularly characterise SIV strains from wild-living *Colobus* and *Cercopithecus*

monkeys has been demonstrated. The storage solution RNA*later*® allows for RNA extraction even when samples have been stored in less than ideal conditions. However, RNA is extremely fragile and can be easily destroyed by RNAses during the extraction procedure. If the faecal sample has already been defrosted several times for other extractions, the RNA quality may be reduced. This RNA frailty reprsents probably one of the main reasons for failures in viral detection and for inconsistencies in the repeatability of results.

A study conducted on captive sooty mangabeys of known infectious status revealed that the sensitivity of antibody detection was significantly greater in urine (96%; Confidence Interval (CI): 87 to 99%) than in faeces (16%; CI, 9 to 27%). By contrast, the sensitivity of vRNA detection was much greater in faeces (50%; CI 32 to 69) than in urine (2%; CI, 0 to 12%) [217]. Hence, a combination of urine and faecal sample analysis would be most useful for field studies, with urine antibody determination allowing prevalence determination and faecal vRNA amplification allowing molecular confirmation and phylogenetic analyses. It may be difficult to collect urine from relatively small arboreal monkeys, therefore its feasibility would need to be tested. Moreover, antibody concentration in urine may be highly variable, as urine density varies significantly at different times of the day. In a captive situation, urine sampling can be standardized, but such conditions may be difficult to reproduce in the wild.

The study on captive sooty mangabeys also revealed that systemic and faecal viral loads are positively correlated and that the ability to detect and amplify vRNA from faecal samples depends on the viral load in the plasma [217]. Plasma viral-load levels may differ in different species. This may explain why we did not succeed in isolating RNA in some species possibly infected with SIV. western red colobus monkeys poorly adapt to a captive environment, therefore there are currently no colonies available for such comparative analysis, hence the SIVwrc viral load in blood samples cannot be assessed. Conversely, *Cercopithecus* species like greater spot-nosed monkeys, Diana monkeys or Campbell’s monkeys are successfully kept in captivity, therefore it would be useful to test and compare SIV detection in blood, urine and faecal samples to assess the feasibility of a non-invasive approach for SIV molecular epidemiological studies in these species in the wild.

## 11.2 SIV epidemiology, social structure and behavioural ecology of non-human primates

Studying animals in a captive setting has the obvious advantage of allowing easy collection of faecal samples. Blood can be regularly taken for good quality DNA and RNA and individuals' behaviour can be easily observed. Copulations, affiliative and agonistic behaviour represent opportunities for SIV transmission that can be quantified and assigned to specific individuals, when the members of an artificially created social group are confined in a limited space. Similarly, offspring can be monitored, parents identified and the likelihood of vertical viral transmission assessed. Zoos and research centres try to keep primates in groups, mirroring wild-living societies in size, sex ratio and patterns of individual migration (for example by exchanging animals between zoos). However, other factors like competition for territory and food resources, population range or population density, and especially predation pressure are more difficult to reproduce. Alliances, hierarchy formation and group interactions can be different in a captive setting, because primates are exposed to altered social and environmental pressures. Some of the differences between a captive and a wild social group reside in the possibility for the latter to interact with sympatric primate species, to actively defend the territory against intruders from the same or from a different species and to allow males or female members to migrate to another social group. These parameters play an important role in the extent and patterns of transmission of a virus which can spread sexually and vertically, but also by bites or mucosal contact with blood or infected wounds. It is therefore obvious that we must be cautious when interpreting SIV prevalence results derived from data extrapolated from captive animals, because they may not reflect the actual prevalence in the wild. Studying NHPs in their natural habitat represents therefore the best approach to obtain accurate results.

The only exclusive advantage of a captive setting is related to the opportunity to monitor the progress of SIV infection. In general, animals tend to live longer, often beyond their average natural lifespan. Diseases with a long incubation period have a chance to develop and can therefore be studied. However, the scientific value will be limited to the understanding of the development of a normally apathogenic and silent disease, which may manifest itself very rarely in a natural setting. Monitoring the

progress of a disease in the wild, especially in dense tropical forests, has its obvious constraints. Not only are weak or ill individuals more easily killed by predators, but it is also much more difficult to distinguish between opportunistic infections caused by immunodeficiency and natural causes of death.

One of the advantages of studying a disease which is sexually transmitted relies on the fact that its spreading pattern is confined to the individual and the interaction he/she establishes with other individuals. It is not related to host density or crowding or other more ‘anonymous’ factors like contracting parasites from walking on the ground. A sexually transmissible disease moves about according to host partner choice and it represents an alternative or complementary method in the field to track species specific behaviour, mating and social structure as well as the individual perimeter of social interactions. SIV is a virus which has a high rate of mutation and turn-over, therefore it will ‘tag’ individuals in different ways.

This peculiarity was exemplified in the study of the two neighbouring red colobus populations from the Taï Forest (Chapter 6). The exceptional case of a sexually mature male moving from one group to the neighbouring one could be traced thanks to the genetically diverse virus he was infected with. The intragroup viral genetic diversity was low, but the intergroup diversity was higher. These results are suggestive of a higher viral transmission within a social group than between different groups. If there is little evidence of viral exchange between groups, sub-adults leaving the natal group may not be infected yet with the virus. For the moment, these are only speculations and further studies are needed to confirm this. The relatively high SIV prevalence in this species and the low genetic diversity within the virus population of each social group may reflect a promiscuous mating system, a hypothesis supported by behavioural studies conducted on these and other red colobus populations.

It has been demonstrated that primate lineages characterised as being more promiscuous exhibited higher white blood cells counts. [263, 266]. Thus, mating and social structures are also crucial components in the understanding of the emergence and control of new diseases in wildlife.

Given the previously discussed technical difficulties encountered in the field and in the lab, we were not able to provide a reliable and clear picture of the SIV prevalence status in every species investigated. Nevertheless, several hypothesis of

SIV prevalence have been formulated and these are analysed and discussed in detail in Chapter 9.

## 11.3 Could the West African chimpanzee (*Pan troglodytes verus*) be infected with SIV?

To date, SIVcpz has only been isolated as a naturally occurring infection in two subspecies of chimpanzee, *Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*. SIVcpz prevalence rates in wild populations have been estimated to be between 4% and 35% [183, 321, 384]. Naturally occurring SIVcpz infection has not yet been detected in the remaining two subspecies, despite screening of about 200 wild-caught *Pan troglodytes verus* over 1700 captive chimpanzees [303, 362], 28 wild-living *P. t. verus* [323] and 78 wild-living *Pan troglodytes vellerosus* chimpanzees [183, 384]. However, the number of wild-living *P. t. verus* and *P. t. vellerosus* tested is quite limited. Similarly, past investigations failed to isolate SIV in gorillas, because only a few samples were analysed [223]. Recently, SIVgor has been isolated from wild-living western lowland gorillas (*Gorilla gorilla gorilla*) in Cameroon after the collection of more than 200 faecal samples [386].

These results suggest that more extensive surveys should be also conducted in wild-living *P.t.verus*, although it may be difficult to isolate the virus today, given the decreasing number of individuals inhabiting the West African forests. The majority of known populations is currently confined to Guinea, with small populations in Sierra Leone, Liberia and Côte d'Ivoire. Only relict populations are found in Mali, Ghana and Senegal, and the subspecies is extinct in six countries (Gambia, Guinea-Bissau, Burkina, Togo, Benin and western Nigeria) [47, 48, 150].

Data obtained from wild-caught and captive born chimpanzees should be taken cautiously. The absence of SIVcpz observed in captive *P. t. verus* may reflect the fact that these animals were captured as infants and therefore their SIV prevalence may be lower [303]. The frequency of vertical transmission in chimpanzees is not known and only a few studies have been conducted on it. The occurrence of early SIV infection in young chimpanzees has been demonstrated in 4 out of 79 wild-caught juvenile *P. t. troglodytes* [73, 290]. Conversely, data from one naturally SIVcpz-infected breeding female suggested that vertical transmission of SIV

may be rare [362]. This is also true for other primate species that are naturally infected with SIV, e.g., African green monkeys and sooty mangabeys [111, 295], and humans. In fact, only about one third of HIV-1-infected women transmit the virus to their infants in the absence of antiretroviral therapy [297, 315].Thus, studies of captive orphans are unlikely to yield accurate estimates of SIVcpz prevalence rates in the wild [257].

In our study, only 5 blood samples collected from wild-living *P. t. verus* were tested and the weakly positive serological results obtained in 3 of them could not be confirmed by PCR analysis. A wide range of universal as wells as species-specific SIV primers were tested on these samples, notably primers amplifying SIVwrc in western red colobus monkeys, a species highly preyed by chimpanzees [31].

Since the INNO-LIA™ HIV I/II Immuno assay gave only weakly positive signals, we cannot exclude that an unspecific antibody cross-reaction might have occurred providing false positive results. Furthermore, since we tested a wide variety of primers on good quality DNA material extracted from blood samples and no SIV positive result was obtained, it is unlikely that these 5 chimpanzees were infected with a lentivirus. However, by further characterising SIVs potentially infecting other species preyed by the chimpanzees or simply other sympatric species, we may be able to design additional and more specific molecular tools and re-test the samples. We cannot therefore exclude for the moment that *P. t. verus* may be infected with a highly divergent virus.

The lack of evidence of SIV infection in *P. t. verus* (but also in *P. t. vellerosus*) suggests a recent introduction of SIV into chimpanzees following subspecies divergence. This hypothesis is consistent with the evolutionary history of *P.t.troglodytes* and *P.t.schweinfurthii*, which both diverged more recently from a common ancestor shared by only these two subspecies [114, 133, 247].

Additional hypotheses related to the role of restriction factors as an intrinsic barrier to retroviral replication have been formulated, although it is not known yet how the degree of antiviral activity conferred by TRIM5α relates to resistance or sensitivity to infection at the organism level [176].

Finally, similarly to chimpanzees from West-central and East Africa [242, 400], *Pan troglodytes verus* is known to hunt other primates for food. This might have been - and still represents today - an opportunity for cross-species transmission and recombination of SIVs. Given the known preference for colobus monkeys, and given the relatively high prevalence of SIVwrc in western red colobus monkeys determined in the frame of this thesis, chimpanzees are at risk for SIV and other infections from these species.

In addition, similarly to *P.t.troglodytes*, *P.t.verus* has always been under human hunting pressure. The possibility that people hunting the chimpanzee from West Africa may be at risk of getting in contact with a potential SIVcpz*Ptv* virus cannot be excluded.

All prerequisites are united here to keep the western chimpanzee population under investigation.

## 11.4 SIV cross-species transmission or virus-host co-evolution? An example from the Colobinae subfamily

The evolutionary forces that shape SIV diversity are still unclear. It is also unknown whether SIV is an ancient infection that has been co-evolving with its primate hosts for millions of years, or whether the virus may have arrived more recently and swept across already established primate lineages. In some cases, closely related monkey species such as l'Hoest and sun-tailed monkeys or the four species of African green monkeys (genus *Chlorocebus*) harbour closely related SIVs. This has led to the hypothesis that some SIVs may have been coevolving with their hosts for an extended period of time, whereby the ancestors of today's monkey species were infected with ancestors of their respective SIVs [3, 21, 168]. However, this hypothesis appears controversial. According to molecular clock studies that estimate the depth of the primate lentivirus phylogeny, SIVs evolved several orders of magnitude more recently than the ancestors of the extant monkey species [158, 206, 333, 341]. A recent study showed that well-resolved phylogenies, based on full-length African green monkey (AGM) mitochondrial genomes and seven published SIVagm genomes, were incongruent in their topologies. Therefore, no phylogenetic support has been found for an ancient SIV infection followed by co-divergence [402].

There are also many examples of cross-species transmission [29] and recombination [14, 87, 168, 350], indicating that many SIVs have spread between different primate species [168]. Although it seems clear today that the overall pattern of the SIV and host phylogenies cannot be reconciled with a simple history of co-divergence [59], certain groups of SIVs and their hosts suggest a shared evolutionary history. An alternative mechanism by which pathogen and host phylogenies could resemble each other is preferential host-switching [59]. This model suggests that viruses are more likely to be transmitted between hosts with less phylogenetic distance separating them. This will lead to a viral phylogeny, among closely related species, that is similar to the host tree, even in the absence of a shared history. Another hypothesis refuting an ancient SIV infection followed by co-divergence among AGM species speculates that immune factors such as the APOBEC proteins [34, 328] and TRIM5α [360] may have prevented the more distantly related *Cercopithecus* monkeys from becoming infected with SIVagm and similarly blocked

the introduction of SIV from non-AGM species into the AGMs. Although findings thus far suggest that the SIV virus was not a relevant force in the ancient evolution of these proteins, intrinsic immunity factors may have been crucial in shaping the distribution of SIV across the range of the African primates it infects [402] .

In order to better understand virus-host dynamics and evolution, it is important to further document new SIV strains and to establish their phylogenetic relationship with other known SIVs as well as their congruence with host genomic evolution. Furthermore, elucidating the different origins of mosaic genomes will help to better understand the role of ancestral strains of the HIV that are causing the current HIV infection and AIDS pandemic.

In our studies, we showed that two western subspecies of red colobus (*Piliocolobus badius*) found in The Gambia and Côte d'Ivoire are both infected with closely related species-specific SIVs, designated SIVwrc. The SIV full-length genome sequences we obtained were also most closely related to the SIVs from the l'Hoest lineage (comprising SIVs from *Cercopithecus lhoesti*, *Cercopithecus solatus* and *Mandrillus sphinx*). We also sequenced the SIV genome of an olive colobus (*Procolobus verus*) from Côte d'Ivoire. Phylogenetic analyses showed that SIVwrc and SIVolc are distinct SIV lineages, but that they are closely related at the genomic level. Interestingly, in *gag* and part of the *pol* region of the SIV genome, SIVwrc and SIVolc were also related to SIVcol isolated from a mantled guereza (*Colobus guereza*) from Cameroon, another fully characterised SIV in the subfamily Colobinae.

With these studies we provided additional information on SIVs infecting African species from the Colobinae subfamily. Our results clearly show that the evolution of their SIVs is linked to that of the Cercopithecinae, but since many species and subspecies of *Colobus* and *Piliocolobus* have not yet been investigated for SIV infection, it remains difficult today to detail the chronology of events of cross-species transmission and/or co-evolution that resulted in the viruses isolated from present day Colobines. Many questions remain unsolved. Past and present geographic distribution of NHPs needs to be considered in order to better understand and explain the potential of SIV cross-species transmission. Geography and behaviour would provide opportunities for the transmission of SIVs from one species of *Colobus* to another or between *Cercopithecus* and *Colobus* species. Moreover, since SIV is predominantly a sexually transmitted virus and since hybridisation areas

cannot be excluded, sexual encounters between different species may have facilitated transmission across equatorial Africa. By isolating SIVs from West-Central and East African regions, we may be able to answer this question.

The geographical range of the colobines and the cercopithecines show a great deal of overlap in Africa and Asia, but the present day distribution of cercopithecines is the more extensive. Fossil evidence suggests, however, that Miocene and Pleiocene colobines inhabited a larger geographical area, and that many of these earlier colobines lived in relatively open woodland and were at least partly terrestrial compared to the extant colobines, which are predominantly arboreal and inhabiting moist lowland tropical forests [187, 269].

Figure 11.1 shows today's geographic distribution of *Procolobus*, *Colobus* and *Piliocolobus* species. Of the three distinct groups of African colobines, black-and-white colobus monkeys (*Colobus*) have the most continuous distribution, with five commonly recognized species dispersed throughout equatorial Africa [142, 273]. Red colobus monkeys (*Procolobus* [*Piliocolobus*]) are also distributed across equatorial Africa, from The Gambia to Zanzibar, but populations are more fragmented and their taxonomy is unstable with little consensus on the number of species that should be recognized. The olive colobus monkey (*Procolobus* [*Procolobus*]) is monotypic and restricted to the Guinean costal forests of West Africa, ranging from Sierra Leone to Nigeria [272].

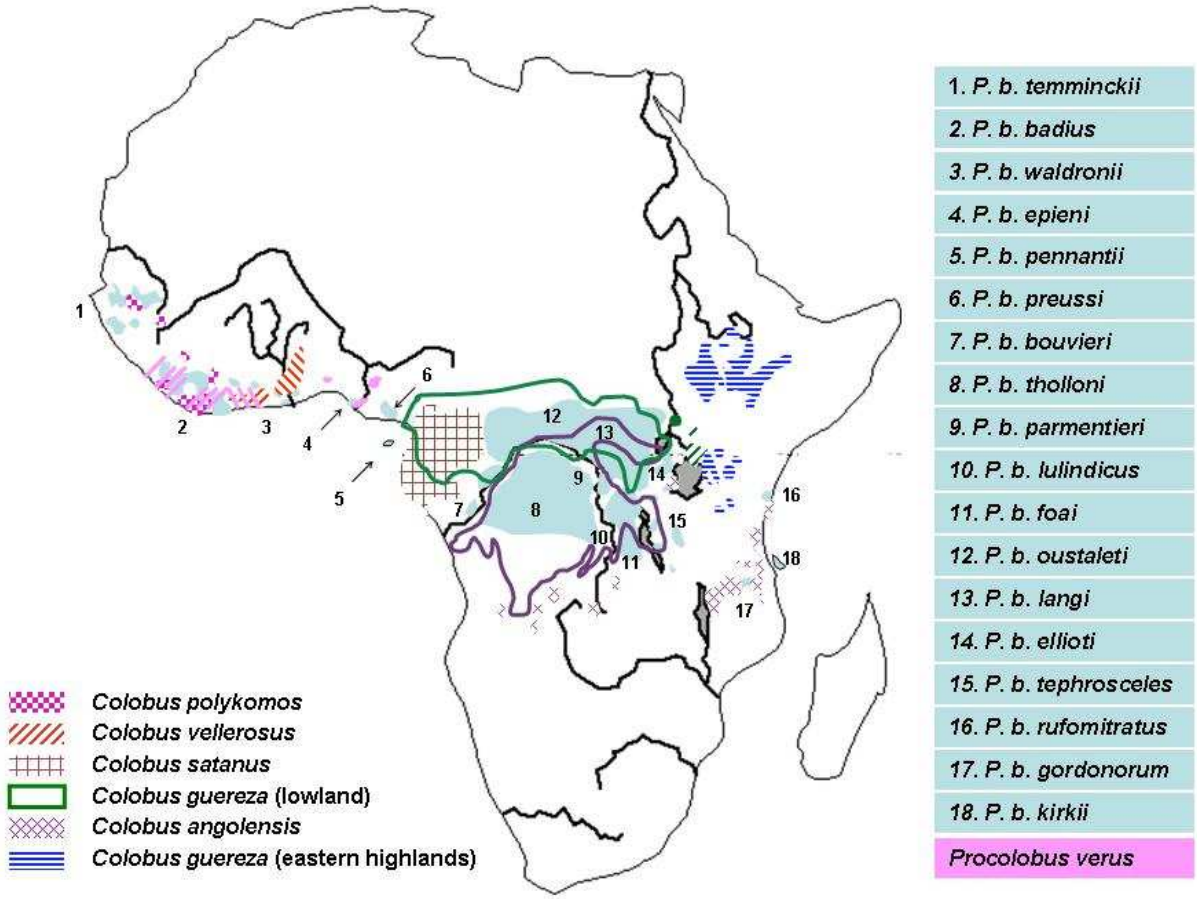



Figure 11.1: Geographic distribution of the African Colobinae. Classification and distribution follows [142, 272]

Figure 11.2 shows the Cercopithecinae and Colobinae species found, to date, to be infected with SIVs, bearing phylogenetic relationships. *Piliocolobus badius temminckii* and *Piliocolobus badius badius* are the western-most subspecies of the genus *Piliocolobus* and their distributions as well as that of *Procolobus verus* overlap neither with that of black and white colobus, found in West-central Africa, nor with that of the mandrills or drills, sun-tailed or l'Hoest monkeys, which are found as far as the most eastern parts of Central Africa. There is clearly a missing link between these infected NHP species.

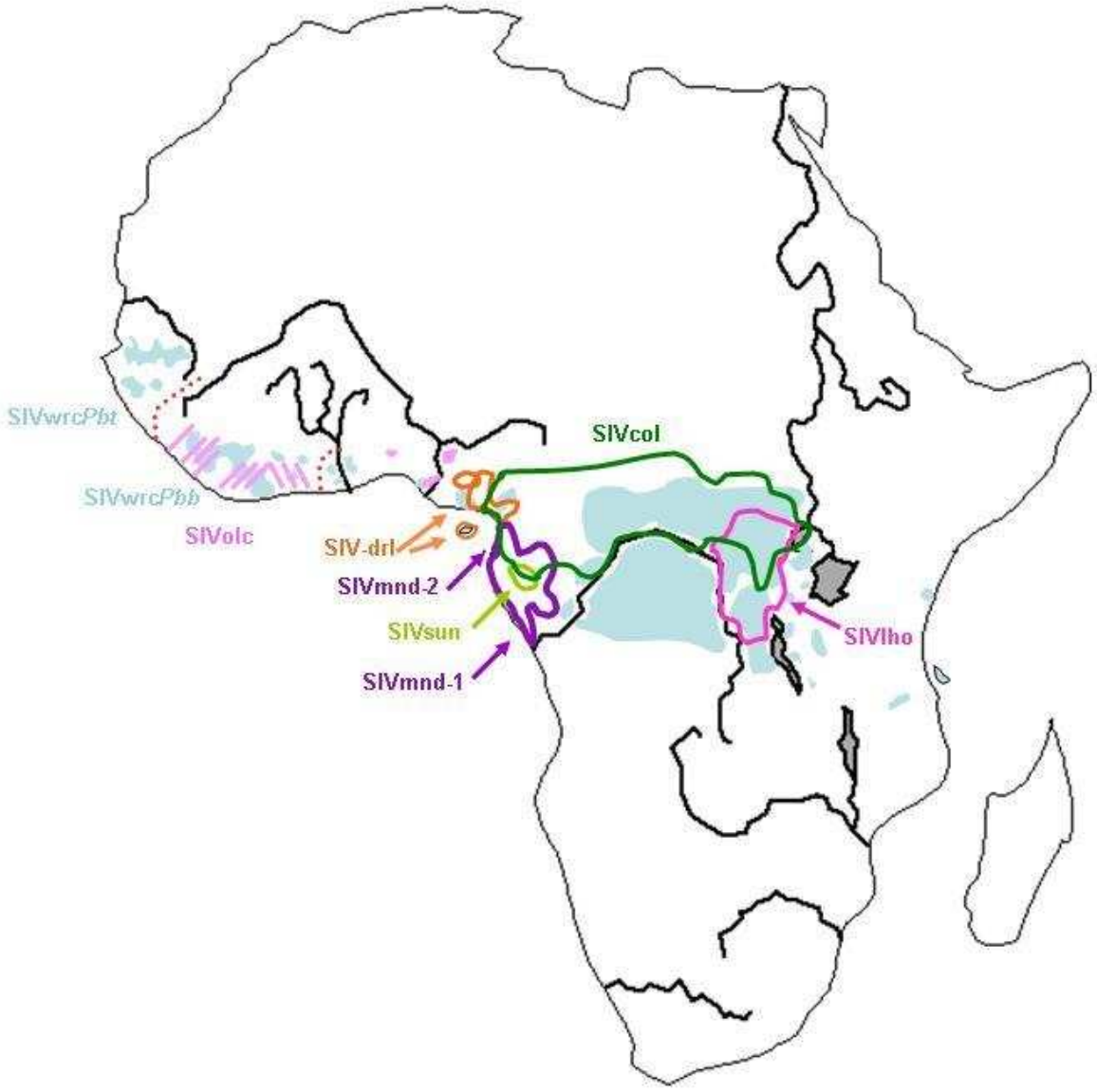



Figure 11.2: Geographic distribution of African NHP species

Geographic distribution of NHP species wheose respective SIVs bear some phylogenetic relationship (adapted from [187]). SIVlho lineage to which SIVwrc*Pbt* and SIVwrc*Pbb* are related, comprises SIVlho from L'Hoest monkeys, inhabiting mountain and lowland forests along the right bank of the river Congo, SIVsun from sun-tailed monkeys, inhabiting the Fôret des Abeilles, a restricted area of about 10300km$^2$ in Gabon and SIVmnd-1 from mandrills inhabiting Gabon, south of the Ogoué river. The SIVmnd-2 lineage which comprises SIVmnd-2 from mandrills living in the region south of the Sanaga river in Cameroon and north of the Ogoué river in Gabon and SIVdrl from drills occurring on both the mainland (from south-eastern coastal Nigeria through south-western Cameroon to the Sanaga river) and on the island of Bioko. *Colobus guereza* infected with SIVcol inhabits the lowland areas of tropical West-central Africa.

In accordance with fossil, nuclear DNA and complete mitochondrial genome analysis, the divergence date estimated (with approximate 95% confidence intervals) for the Cercopithecinae - Colobinae split was 16.2 Mya [305] (Figure 11.3). The most parsimonious explanation for colobine origins is that they first evolved in Africa, and then dispersed into Asia less than 10 milion years ago [305] and, to date, no Asian NHPs have been found to be naturally infected with SIV [173, 399].

Speciation probably occurred during the Pliocene-Pleistocene (the last 5.3 million years), a time characterised by important changes in African faunal evolution mediated by changes in climate or shifts in climate variability [89]. These alternating Pleistocene intervals of glacial maxima have been used to infer recent origins for the living colobus monkeys [141, 167, 273, 361]. The oldest specimens attributable to an extant lineage were found in early Pleistocene deposits [207]. A recent study shows that the modern African colobines had started to diversify much earlier than this [374], and are consistent with the finding that this radiation had begun by the Late Miocene [357]. All three colobus clades had differentiated by the end of that epoch, and by the Pliocene and early Pleistocene many of the extant species lineages were already present [357].

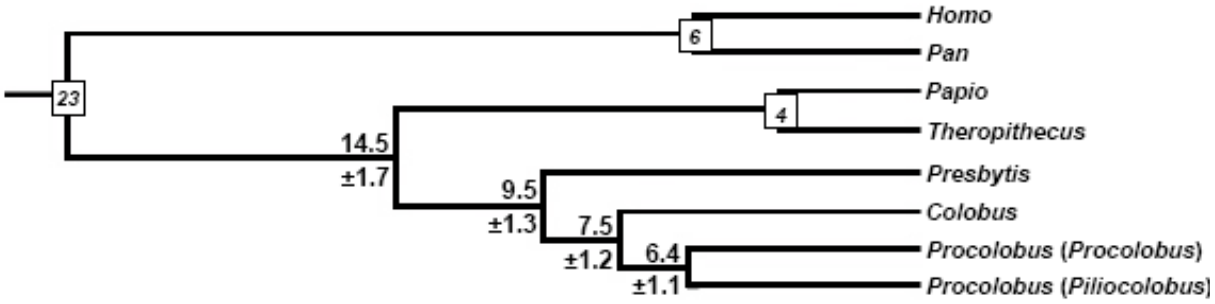



Figure 11.3: Catarrhine mitochondrial likelihood and Bayesian tree based on 3,831 base pairs of DNA sequence from the NADH3, NADH4, NADH4L, and NADH5 genes. All nodes supported by bootstrap values > 85 and posterior probabilities > 0.90. Divergence date estimates (Mya) from penalized likelihood shown with two standard deviations. Calibration points are boxed and italicized. *Cebus* was used as the outgroup taxon. Classification follows [142]. Courtesy of N. Ting.

Additional information on the origin and age of SIV in colobus monkeys will help to better assess virus-host dynamics and evolution by comparing it with the Colobinae divergence and speciation dates.

Phylogenetic hypothesis using analysis of morphology, pelage and vocalization places the olive colobus and the red colobus as sister taxon to the exclusion of the black and white colobus [142, 273, 361] (Figure 11.4). This phylogenetic proximity has also been observed between SIVolc and SIVwrc. Recent molecular analysis of the red colobus group revealed a paraphyly in *P. b. badius* with respect to *P. b. temminckii*, indicating that these animals may have shared gene flow until very recently, which is plausible given that their ranges are poorly documented and possibly still overlap [374]. Similarly, their respective SIVs (SIVwrc*Pbt* and SIVwrc*Pbb*) are paraphyletic.

Although a taxonomically comprehensive molecular survey of the African colobines has been conducted, additional work is required to elucidate the evolution of the red colobus group. Mitochondrial relationships were congruent with those based on pelage and vocalization data with respect to the grouping of *P. b. gordonorum* with *P. b. kirkii*, *P. b. badius* with *P. b. temminckii*, and *P. b. pennantii* with *P. b. preussi*. However, the *P. b. pennantii*/*P. b. preussi* pair does not seem to have any affinity with the *P. b. badius*/*P. b. temminckii* pair; but they rather seem to be more closely related to some, if not all, of the taxa found further east. *P. b. parmentieri* groups more distantly from most of the Central African forms.

It would be interesting to investigate these species for the presence of SIV infection and to assess whether similar groupings are also present in their SIV phylogeny. This will not only help us to elucidate the pattern of spread of SIV in colobines, but also to better trace the origin and evolution of SIV across the African continent.

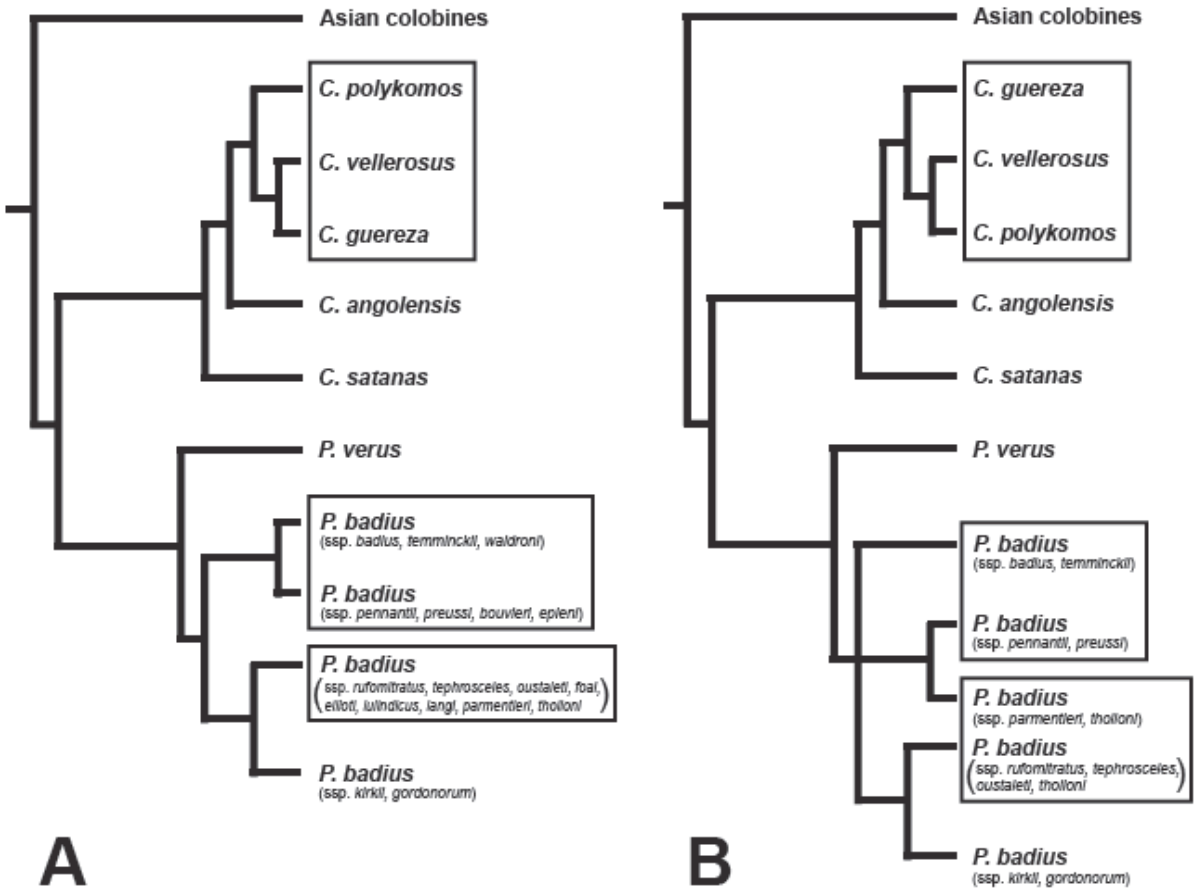



Figure 11.4: Cladograms showing incongruence between phenotypic and mitochondrial phylogenetic hypotheses among the extant African colobines. A – Phylogenetic hypotheses based on morphology, pelage, and vocalizations [142, 273, 361]. B – Mitochondrial relationships inferred by Ting [374]. Boxed taxa highlight the differences between the cladograms. Notice differences between the sister taxon to *C. vellerosus* and the phylogenetic groupings of the *P. badius* subspecies. Some subspecies of red colobus were not sampled. Courtesy of N. Ting.

## 11.5 The bushmeat trade: a conservation and a public health issue

Hunting and handling of bushmeat places both people and wildlife at risk. Despite only limited testing, it is already known that more than 1% of Cameroonians hunters are infected with Human T-lymphotropic viruses (HTLVs) [412] and between 1% and 5% are infected with wild primate spumaviruses or simian foamy virus (SFV) [250, 413]. Dramatic consequences have already emerged from the direct exposure to primate blood infected with SIV, exemplified by the current HIV/AIDS pandemic, but attempts to isolate SIV from human blood remain elusive [178].

Very few studies aiming at isolating retroviruses from hunters or whomever comes into contact with primate blood have been conducted, and mainly in Cameroon where a plethora of different SIVs have been detected and isolated from bushmeat found at local markets [287].

In Liberia and Sierra Leone, rare HIV-2 subtypes have been identified to be very closely related to SIVsmm strains circulating in the same regions [61, 124]. In one case SIVsmm isolated from a pet sooty mangabey was most closely related to an HIV-2 isolated from a man living in the same village where this monkey was kept [62].

In The Gambia and especially in Côte d'Ivoire, where bushmeat trade and consumption has been documented [54], no epidemiological survey of retroviruses infecting the populations surrounding the park has been conducted. It would be of great importance to screen these populations as well as to lead a parallel survey on disease risk exposure and perception of risk of contracting an infection by handling and consuming bushmeat.

People in contact with wild animals, and particularly NHPs, are acutely at risk of zoonoses and should take steps to avoid infections. Taking these steps implies that people perceive this contact as a risk to their health. Given that little is known about many pathogens, their effects, and the number of contact events required for a zoonotic disease to become established in a human population, it is impossible to assess the risk to the public and the likelihood that individuals will perceive the risk as sufficient to alter their behaviour. The threat posed by an infection may not outweigh the potential value of bushmeat as a source of revenue. In retrovirus infections, there is often a long delay before the onset of symptoms, making a direct

association between the causative event and the actual disease difficult, thus allowing further viral transmission before the disease is recognised. Moreover, in many ethnic groups, especially in central Africa, it is often believed that illness has supernatural rather than biological origins [406]. Therefore, dealing with the roots of the emerging infectious diseases involves dealing with cultural, political, economic and ecological complexities.

Health education could help people to become aware of the risks they encounter when hunting and butchering NHPs. However, the limits of relying solely on this approach must be acknowledged. In fact, until the supply of domestic animal substitutes rises to meet the demand for animal protein, rural families living in close proximity to wildlife will continue to hunt and consume bushmeat. Food supply, poverty and corruption must be addressed before the practice of harvesting bushmeat can be changed.

Moreover, It is important to ensure that the hunting of threatened species is not inadvertently encouraged or legitimized by promoting safe and healthy hunting [85, 243].There is in fact the risk that a “butcher bushmeat with care” campaign will not only not change bushmeat-harvesting rates, it may actually be seen as an approval of bushmeat hunting and consumption.

In addition, wildlife is increasingly identified as a reservoir of lethal diseases and its image may start to decline in the public eye. We need to be reminded that it is not disease-carrying wildlife that are the problem. It is what we do to wildlife as individuals (by hunting and butchering them) and collectively (by building roads into wildlife habitat and forcing contact with our domestic animals) that provides the pathway for pathogens that Stephen Morse called “viral traffic” to flow [248].

As researchers we have the moral obligation to make sure that scientific findings are communicated simply and clearly. An incorrect assumption concerning the risk of acquiring AIDS from simian bushmeat may result in deliberate killing of monkeys to prevent the spread of AIDS, a disastrous consequence for endangered non-human primates that is likely to have little effect on the AIDS epidemic.

## 11.6 How likely is the emergence of a novel HIV?

SIV has already been isolated in 40 species of African NHPs, but there are reasons to believe that many more species could be infected with this virus. Moreover, numerous viral, host and environmental factors can interact to create opportunities for SIV to evolve into new ecological niches, reach and adapt to new hosts, and spread more easily between them.

Retroviruses are known for their high mutation rate and recombination capabilities. The likelihood of exposure, the rate of exposure and the transmission biology of the virus itself are the first prerequisites for a jump into a new NHP species. The second step is related to the ability of the virus to infect the new host. If the new host displays appropriate cell receptors, i.e. cell receptors that are phylogenetically conserved, then it can be considered 'compatible' for viral transmission. Finally, for a successful species jump to occur, the pathogen would have to be sufficiently transmissible among individuals from the new host population. In Taï National Park, Côte d'Ivoire, and in Abuko Nature Reserve, The Gambia, different NHP species share the same habitat and have been observed to display affiliative as well as agonistic behaviours. The association of different species as an anti-predation strategy is a behaviour frequently observed in the Taï Forest, especially between olive colobus and Diana monkeys. These host interactions represent a potential opportunity for cross-species transmission of viruses and other pathogens through grooming, biting or licking wounds between infected, non-infected or super-infected individuals.

It has been repeatedly reported that most SIVs will replicate in human peripheral blood mononuclear cells (PBMCs). Their ability to replicate *in vitro* in human PBMC or T cell lines has been evoked as a major argument in favour of these viruses infecting the human population exposed. This ability has been documented for SIVcpz [290], SIVsm/SIVmac [23, 279], SIVagm [151, 279], SIVlhoest [151], SIVmnd-1 [23, 300], SIVrcm [23, 127], SIVmnd-2 and SIVdrl [162, 350, 364] . Extensive *in vitro* studies on SIVs properties are needed in order to correctly evaluate the threat for human population following exposure to these viruses. Given the high phylogenetic and molecular relatedness of SIVwrc and SIVolc with the SIVlho lineage, we cannot exclude that these SIVs are also able to infect human PBMCs.

While the potential ability of a virus to infect human PBMCs may not be predictive of its virulence in humans, serologic surveys of humans in regions where red and olive colobus live are necessary to evaluate this possibility. However, viral cross-species transmission is in itself not the only requirement for the generation of epidemics. Other factors are required for HIV adaptation and epidemic spread of SIV in the new human host.

Innate host specific restrictions on viral replication have recently become evident for primate lentiviruses [225, 360]. This may explain why only certain NHP species harbouring simian immunodeficiency viruses gave rise to HIV-1 and HIV-2. Despite frequent human exposure to SIV-infected monkeys in Africa, only eleven known cross-species transmissions to human beings have occurred, and only four resulted in significant human-to-human transmission (HIV-1 groups M and O and HIV-2 groups A and B). Repeated passages of nonhuman viruses to humans do not necessarily result in human to human transmission. However, high rates of transmission will increase the diversity of viruses and sequence variants moving into humans, increase the probability of transmission of a pathogen that can successfully replicate, and ultimately increase the ability of a human-adapted virus to emerge in a more widespread manner. In some cases this process may result in the evolution of a new viral strain [6] and may be a very common mechanism for viral emergence into the human population [143].

In addition, the capability to cross the species barrier and infect a different NHP host is today facilitated by anthropogenic factors like natural habitat fragmentation. Habitat fragmentation increases the contact between different NHP species, facilitating the cross-species transmission of pathogens.

Unfortunately, there is a higher possibility for animal-to-human infection to occur owing to greater exposure, i.e. bushmeat hunting ,but also to a greater chance for onward transmission, once a person has become infected (e.g. sexual contact, iatrogenic factors). In equatorial Africa, the logging industry is in expansion with currently more than 600,000km$^2$ (30%) of central Africa's dense humid forests under logging concessions, and just 12% under protection. Wildlife hunting pressure has increased accordingly, because of easier access to remote forest areas and local market opportunities offered by new logging towns [202]. The construction of new

roads into the forests has also led to an increased human migration, the expansion of villages around the logging concessions and to the development of new social and economic networks (including those of sex workers) supporting the logging industry [408]. A social-epidemiological study conducted around logging concessions in rural Cameroon showed that HIV prevalence reaches 23% of young women between 25 and 34 years old [205]. HIV-1 genetic diversity and distribution were similar to those observed in towns [51, 392], which suggests that the spread of HIV in these rural areas result from numerous introductions of the virus.

In conclusion, the socio-economic changes associated with logging, mining or other industrial activities in remote forest areas, combined with the SIV prevalence and genetic complexity of the virus, suggest that the magnitude of human exposure to SIV is increasing. Hence, the recombination of SIVs and HIV and the potential for dissemination of these new variants cannot be excluded.

Successful crossover infections may have already occurred in low numbers in remote areas of Africa, but because of low population density and isolation, they do not have the opportunity to become epidemic strains and instead become dead-end infections. Ongoing transmission events may also be missed because serologic assays for detecting a broad range of SIVs are lacking or because monitoring is insufficient in populations with high levels of exposure to NHP blood and body fluids. It is therefore imperative to develop highly sensitive and specific serologic assays for the surveillance of SIV infections in humans and the prevention of the emergence of a new HIV epidemic.

# 12. Conclusions and Perspectives

I attempted in this thesis to determine the prevalence and describe the molecular characteristics of the simian immunodeficiency virus infecting several species of non-human primates inhabiting the forests of West Africa, via non-invasive collection methods. I will briefly underline here their significance and suggest foci for future research.

The microsatellite analyses conducted on faecal samples showed some limitations and the results obtained stress the importance of screening additional markers in the future. By screening more microsatellite loci, the genetic variability within and between populations of the same species is likely to be characterised more accurately and fine-scale patterns of group structure, mating system, territory range and dispersal could be explored. Moreover, by investigating paternity and maternity, vertical transmission of SIV could be assessed in sexually immature individuals to further document the relevance of this viral pattern of transmission. Light could also be shed on the degree of bias in dispersal within a species, the dispersal frequencies and distances. Whether males, females or both sexes disperse is highly relevant, because this can have a different impact on the spread of infection agents, as well as on the population genetics of a species. Traditional markers for molecular sex determination in primates were developed on the basis of human sequences and were non-functional in distantly related primate species like the Colobines or Cercopithecines we investigated. However, today, a novel method is available for sex identification in primates [396] that allows the amplification of very short and highly conserved X and Y fragments from all species of primates. This could also be applied to fragmented DNA extracted from non-invasive samples.

In this thesis I demonstrated that western red colobus monkeys are infected with a species specific SIV and represent a substantial reservoir for SIVwrc. This is relevant with respect to the risk of SIV transmission to other sympatric species living in Taï Forest, especially to chimpanzees, which regularly prey on western red colobus, and ultimately toward the human population who also hunts and handles the flesh of these primates.

The results on the SIV genetic diversity obtained within and between the western red colobus groups investigated allowed us to draw some interesting parallels with their social structure and mating system. The relationship between virus diversity and its host should be investigated further. This study should be extended to all members of the two habituated groups and possibly also to other neighbouring groups. All individuals should be sampled in order to better define the host-virus dynamics. Demographic, ecological and behavioural data can significantly contribute to elucidating prevalence and disease transmission and partly helped to explain why SIVs from other NHP species from the Taï Forest could not be isolated. In addition to host genetics research, theoretical virus-host disease dynamics could be modelled to compare and corroborate the results obtained.

One of the major challenges will be that of collecting faecal samples from all age classes in relatively small arboreal monkeys. According to my previous experience in the field, this task is difficult to accomplish even in habituated animals: infants and juveniles tend to hide in the canopy more often than adults and females adopt a more reserved attitude than males. Consequently, sampling non-habituated populations would require great effort. Many more researchers and assistants should be present on the ground to track several groups at a time, although the risk of disturbing the groups may increase.

Another important aspect which necessitates attention is the conservation of the samples collected while in the field. Despite the significant practical improvements achieved with the advent of RNA*later*®, the storage of faecal samples at room temperature over long periods of time represents a threat for successful analyses. Despite these limitations, interesting data have been obtained and further, more extensive sample collection should be encouraged.

Opportunistic collection of NHP carcasses from the forest floor is also valuable, because, even if a primate species is infected with a divergent virus, blood or tissue extraction guarantees better DNA quality and SIV fragments are more likely to be amplified. Starting from such DNA templates would provide more chances to find species specific consensus regions and therefore amplify SIV genome fragments issued from partially degraded material. However, sampling carcasses must be carried out under strict safety procedures. Given the unknown reasons of death of

the animal, direct contact with the dead body should be avoided in order to prevent the risk of being infected with potentially highly dangerous pathogens.

Given the serological SIV positive results previously obtained by other researchers on Diana monkeys and on western chimpanzees, the frequent association of the Diana monkeys with other sympatric species and the vulnerability of chimpanzees to human hunting pressure, high priority should be given to the investigation of SIV infection and prevalence in these two species in the near future. It is particularly important to pursue the search for SIV in West African chimpanzees because, similarly to the other ape species, the physiological and genetic proximity between the chimpanzee and humans makes our infectious disease interface very thin. Chimpanzee populations have become rare in West Africa, therefore a systematic and comprehensive investigation of a possible SIVcpz infection will require monitoring several sites across those regions. Additionally, the more information on SIV infected monkeys preyed by chimpanzees we acquire, the better the chances to isolate a SIV in the western chimpanzee.

In this thesis four SIV strains (one SIVwrc*Pbt*, two SIVwrc*Pbb*, and one SIVolc) belonging to two different species of the Colobinae subfamily were molecularly characterised and their phylogenetic relationship with strains from other NHP species was determined. It is important to further characterise new SIVs in order to explore their genetic diversity and to better understand the evolutionary history of this virus. Since the length of association between the virus and apes and monkeys is not known, and since the infected primates do not develop immunodeficiency, it has been suggested that some of these monkey species have been infected for millions of years, because many closely related monkey species are infected with closely related viruses. However, if this infection is instead relatively recent, then non-virulence may have evolved over a shorter time frame than previously suggested. This finding could have implications for the future trajectory of HIV disease severity.

Moreover, by further characterising recombinant genomes, we will be able to better assess which SIV strains are more likely to cross the species barrier and ultimately reach humans. Subsequently, understanding which viruses are likely to rapidly evolve in humans, rather than become dead-end hosts, will involve the investigation of a combination of host immunologic and viral evolutionary traits.

SIVwrc infectivity, growth potential and cytotoxic properties should be tested in human PBMC cells and subsequently compared with models of HIV pathogenesis.

One of the major concerns today is related to the seeding of new lentivirus epidemics that may go unchecked because current serological assays may not be able to detect such potentially infectious diseases. Until recently, there were no sensitive and specific diagnostic tools suitable for systematic and comprehensive surveys of SIV infection in wild primate populations. Synthetic peptide based SIV assays covering the V3 region and the immunodominant region of the *env* gene were available, but they were not able to detect and discriminate all SIV lineages identified thus far. Therefore, it is important to develop and validate serological screening assays that are sensitive and specific enough to detect all known and potentially some currently unknown but antigenically similar SIV lineages. The recently developed Luminex technology performs the screening of multiple antigens simultaneously, which would allow the testing of a broader spectrum of SIVs in a considerably shorter amount of time.

To address the question of whether SIVwrc or other SIVs have previously occurred in humans, but have gone undetected, it would be necessary to screen blood samples of the populations surrounding the park, which have come into contact with blood or fluids of NHPs. Because, in general, pathogen emergence is unpredictable, the first line of defence has to be effective surveillance. This requires identification and monitoring of high risk populations, or individuals, or locations.

Finally, more attention should be given to multidisciplinary studies at all stages of the process of a possible virus cross-species transmission. A great understanding has already been acquired of the environmental/anthropogenic changes that drive emergence by affecting wildlife density and virus prevalence. In some African regions risk assessments for pathogen transmission have already been conducted. In Côte d'Ivoire, apart from a study on hunting, trading and consumption of bushmeat conducted by Caspary [54] and the impact of commercial hunting on the monkey populations in the Taï region by Refisch and Koné [310], no other published information is available. In order to predict if the Taï Forest and surrounding areas represents a hotspot for disease emergence, additional studies on bushmeat hunting

and other practices that put humans in contact with NHPs should be conducted. NHP crop raiding, keeping primates as pets, or performing rituals with NHP body parts pertain to this category. The human perception of disease risk and cultural or religious believes would also influence the likelihood of coming into contact with pathogens and may be revealing in areas where ethnically diverse human populations are cohabiting.

Unfortunately, HIV prevalence is increasing in rural areas, hence recombination between newly introduced SIVs and circulating HIVs can pose an additional risk for the outbreak of a novel epidemic. In fact, in the present climate of AIDS pandemic, avian influenza, Ebola and SARS, the question of what launches new epidemics is extremely important. Equally important is the question as to why most animal viruses fail to launch sustained human to human transmissions.

In conclusion, the current HIV-1 pandemic demonstrates that the transmission of NHP lentiviruses into the human population can have unexpected and very serious consequences. Furthermore, additional cross-species transmissions of NHP lentiviruses other than of chimpanzee, gorilla or of sooty mangabey origin may already have happened, just waiting for a chance to become the next global plague.

# 13. References


1. **Aghokeng, A. F., E. Bailes, S. Loul, V. Courgnaud, E. Mpoudi-Ngole, P. M. Sharp, E. Delaporte, and M. Peeters.** 2007. Full-length sequence analysis of SIVmus in wild populations of mustached monkeys (*Cercopithecus cephus*) from Cameroon provides evidence for two co-circulating SIVmus lineages. Virology **360:**407-18.
2. **Aghokeng, A. F., W. Liu, F. Bibollet-Ruche, S. Loul, E. Mpoudi-Ngole, C. Laurent, J. M. Mwenda, D. K. Langat, G. K. Chege, H. M. McClure, E. Delaporte, G. M. Shaw, B. H. Hahn, and M. Peeters.** 2006. Widely varying SIV prevalence rates in naturally infected primate species from Cameroon. Virology **345:**174-89.
3. **Allan, J. S., M. Short, M. E. Taylor, S. Su, V. M. Hirsch, P. R. Johnson, G. M. Shaw, and B. H. Hahn.** 1991. Species-specific diversity among simian immunodeficiency viruses from African green monkeys. J Virol **65:**2816-28.
4. **Allen, M., A. S. Engstrom, S. Meyers, O. Handt, T. Saldeen, A. von Haeseler, S. Paabo, and U. Gyllensten.** 1998. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. J Forensic Sci **43:**453-64.
5. **Apetrei, C., B. Gormus, I. Pandrea, M. Metzger, P. ten Haaft, L. N. Martin, R. Bohm, X. Alvarez, G. Koopman, M. Murphey-Corb, R. S. Veazey, A. A. Lackner, G. Baskin, J. Heeney, and P. A. Marx.** 2004. Direct inoculation of simian immunodeficiency virus from sooty mangabeys in black mangabeys (*Lophocebus aterrimus*): first evidence of AIDS in a heterologous African species and different pathologic outcomes of experimental infection. J Virol **78:**11506-18.
6. **Apetrei, C., and P. A. Marx.** 2004. Simian retroviral infections in human beings. Lancet **364:**137-8.
7. **Apetrei, C., M. J. Metzger, D. Richardson, B. Ling, P. T. Telfer, P. Reed, D. L. Robertson, and P. A. Marx.** 2005. Detection and partial characterization of simian immunodeficiency virus SIVsm strains from bush meat samples from rural Sierra Leone. J Virol **79:**2631-6.
8. **Apetrei, C., D. L. Robertson, and P. A. Marx.** 2004. The history of SIVS and AIDS: epidemiology, phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa. Front Biosci **9:**225-54.

9. **Armstrong, W., L. Calabrese, and A. J. Taege.** 2005. HIV update 2005: origins, issues, prospects, and complications. Cleve Clin J Med **72:**73-8.

10. **Ashford, R. W.** 2000. Parasites as indicators of human biology and evolution. J Med Microbiol **49:**771-2.

11. **Auzel, P., and D. S. Wilkie.** 2000. Wildlife use in Northern Congo:hunting in a commercial logging concession. *In* J. G. Robinson and E. L. Bennett (ed.), Hunting for Sustainability in Tropical Forests. Columbia University Press, New York.

12. **Ayouba, A., P. Mauclere, P. M. Martin, P. Cunin, J. Mfoupouendoun, B. Njinku, S. Souquieres, and F. Simon.** 2001. HIV-1 group O infection in Cameroon, 1986 to 1998. Emerg Infect Dis **7:**466-7.

13. **Ayouba, A., S. Souquieres, B. Njinku, P. M. Martin, M. C. Muller-Trutwin, P. Roques, F. Barre-Sinoussi, P. Mauclere, F. Simon, and E. Nerrienet.** 2000. HIV-1 group N among HIV-1-seropositive individuals in Cameroon. Aids **14:**2623-5.

14. **Bailes, E., F. Gao, F. Bibollet-Ruche, V. Courgnaud, M. Peeters, P. A. Marx, B. H. Hahn, and P. M. Sharp.** 2003. Hybrid origin of SIV in chimpanzees. Science **300:**1713.

15. **Balzarini, J., E. De Clercq, and K. Uberla.** 1997. SIV/HIV-1 hybrid virus expressing the reverse transcriptase gene of HIV-1 remains sensitive to HIV-1-specific reverse transcriptase inhibitors after passage in rhesus macaques. J Acquir Immune Defic Syndr Hum Retrovirol **15:**1-4.

16. **Barlow, K. L., A. O. Ajao, and J. P. Clewley.** 2003. Characterization of a novel simian immunodeficiency virus (SIVmonNG1) genome sequence from a mona monkey (Cercopithecus mona). J Virol **77:**6879-88.

17. **Barnett, S. W., K. K. Murthy, B. G. Herndier, and J. A. Levy.** 1994. An AIDS-like condition induced in baboons by HIV-2. Science **266:**642-6.

18. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-71.

19. **Beer, B., M. Peeters, S. G. Norley, G. van der Groen, L. K. Fransen, H. Willkommen, and R. Kurth.** 1995. Failure to infect lower primate species with SIVcpz. Aids **9:**527-8.

20. **Beer, B. E., E. Bailes, G. Dapolito, B. J. Campbell, R. M. Goeken, M. K. Axthelm, P. D. Markham, J. Bernard, D. Zagury, G. Franchini, P. M. Sharp, and V. M. Hirsch.** 2000. Patterns of genomic sequence diversity among their simian immunodeficiency viruses suggest that L'Hoest monkeys (*Cercopithecus lhoesti*) are a natural lentivirus reservoir. J Virol **74:**3892-8.

21. **Beer, B. E., E. Bailes, R. Goeken, G. Dapolito, C. Coulibaly, S. G. Norley, R. Kurth, J. P. Gautier, A. Gautier-Hion, D. Vallet, P. M. Sharp, and V. M. Hirsch.** 1999. Simian immunodeficiency virus (SIV) from sun-tailed monkeys (*Cercopithecus solatus*): evidence for host-dependent evolution of SIV within the *C. lhoesti* superspecies. J Virol **73:**7734-44.

22. **Beer, B. E., E. Bailes, P. M. Sharp, and V. M. Hirsch.** 1999. Diversity and evolution of primate lentiviruses, p. 460-474. *In* C. L. Kuiken, B. Foley, B. Hahn, B. Korber, F. McCutchan, P. A. Marx, J. W. Mellors, J. I. Mullins, J. Sodroski, S. Wolinsky, and B. Karber (ed.), Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.

23. **Beer, B. E., B. T. Foley, C. L. Kuiken, Z. Tooze, R. M. Goeken, C. R. Brown, J. Hu, M. St Claire, B. T. Korber, and V. M. Hirsch.** 2001. Characterization of novel simian immunodeficiency viruses from red-capped mangabeys from Nigeria (SIVrcmNG409 and -NG411). J Virol **75:**12014-27.

24. **Berger, E. A., R. W. Doms, E. M. Fenyo, B. T. Korber, D. R. Littman, J. P. Moore, Q. J. Sattentau, H. Schuitemaker, J. Sodroski, and R. A. Weiss.** 1998. A new classification for HIV-1. Nature **391:**240.

25. **Berger, E. A., J. D. Lifson, and L. E. Eiden.** 1991. Stimulation of glycoprotein gp120 dissociation from the envelope glycoprotein complex of human immunodeficiency virus type 1 by soluble CD4 and CD4 peptide derivatives: implications for the role of the complementarity-determining region 3-like region in membrane fusion. Proc Natl Acad Sci U S A **88:**8082-6.

26. **Berry, N., C. Davis, A. Jenkins, D. Wood, P. Minor, G. Schild, M. Bottiger, H. Holmes, and N. Almond.** 2001. Vaccine safety. Analysis of oral polio vaccine CHAT stocks. Nature **410:**1046-7.

27. **Bibollet-Ruche, F., E. Bailes, F. Gao, X. Pourrut, K. L. Barlow, J. P. Clewley, J. M. Mwenda, D. K. Langat, G. K. Chege, H. M. McClure, E. Mpoudi-Ngole, E. Delaporte, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 2004. New simian immunodeficiency

virus infecting De Brazza's monkeys (Cercopithecus neglectus): evidence for a cercopithecus monkey virus clade. J Virol **78:**7748-62.

28. **Bibollet-Ruche, F., C. Brengues, A. Galat-Luong, G. Galat, X. Pourrut, N. Vidal, F. Veas, J. P. Durand, and G. Cuny.** 1997. Genetic diversity of simian immunodeficiency viruses from West African green monkeys: evidence of multiple genotypes within populations from the same geographical locale. J Virol **71:**307-13.

29. **Bibollet-Ruche, F., A. Galat-Luong, G. Cuny, P. Sarni-Manchado, G. Galat, J. P. Durand, X. Pourrut, and F. Veas.** 1996. Simian immunodeficiency virus infection in a patas monkey (*Erythrocebus patas*): evidence for cross-species transmission from African green monkeys (*Cercopithecus aethiops sabaeus*) in the wild. J Gen Virol **77 ( Pt 4):**773-81.

30. **Blancou, P., J. P. Vartanian, C. Christopherson, N. Chenciner, C. Basilico, S. Kwok, and S. Wain-Hobson.** 2001. Polio vaccine samples not linked to AIDS. Nature **410:**1045-6.

31. **Boesch, C.** 1994. Chimpanzees-red Colobus monkeys : a predator-prey system. Behaviour **47:**1135-1148.

32. **Boesch, C., and H. Boesch-Achermann.** 2000. The Chimpanzees of the Taï Forest: Behavioural ecology and evolution. Oxford University Press, Oxford.

33. **Boesch, C., and H. Boesch-Achermann.** 1989. Hunting behavior of wild chimpanzees in the Tai National Park. Am J Phys Anthropol **78:**547-73.

34. **Bogerd, H. P., B. P. Doehle, H. L. Wiegand, and B. R. Cullen.** 2004. A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor. Proc Natl Acad Sci U S A **101:**3770-4.

35. **Bonhomme, M., A. Blancher, and B. Crouau-Roy.** 2005. Multiplexed microsatellites for rapid identification and characterization of individuals and populations of Cercopithecidae. Am J Primatol **67:**385-91.

36. **Bowen-Jones, E., and S. Pendry.** 1999. The threat to primates and other mammals from the bush meat trade in Africa, and how this threat could be diminished? Oryx **33:**233-246.

37. **Bradley, B. J., and L. Vigilant.** 2002. False alleles derived from microbial DNA poses a potential source of error in microsatellite genotyping of DNA from faeces. Molecular Ecology Notes **2:**602-605.

38. **Bshary, R.** 2001. Diana monkeys, *Cercopithecus diana*, adapt their anti-predator response behaviour to human hunting strategies. Behav. Ecol. Sociobiol.**:**251-256.

39. **Bshary, R.** 1995. Rote stummelaffen, Colobus badius, und Dianameerkatzen, *Cercopithecus diana*, im Taï-Nationalpark, Elfenbeinküste: Wozu assoziierren sie? PhD Thesis. Ludwig-Maximilian- Unviersität München, München.

40. **Bshary, R., and E. R. No.** 1997. Red colobus and Diana monkeys provide mutual protection against predators. Anim Behav **54:**1461-1474.

41. **Bshary, R., and R. Noë.** 1997. Anti-predation behaviour of red colobus monkeys in the presence of chimpanzees. Behav Ecol Sociobiol **41:**321-333.

42. **Buchan, J., S. Alberts, J. Silk, and J. Altmann.** 2003. True paternal care in a multi-male primate society. Nature **425:**179-181.

43. **Burke, D. S.** 1997. Recombination in HIV: an important viral evolutionary strategy. Emerg Infect Dis **3:**253-9.

44. **Bushman, F., M. Lewinski, A. Ciuffi, S. Barr, J. Leipzig, S. Hannenhalli, and C. Hoffmann.** 2005. Genome-wide analysis of retroviral DNA integration. Nat Rev Microbiol **3:**848-58.

45. **Bushmeat Crisis Task Force.** 2000. Bushmeat. A wildlife crisis in West and central Africa and around the world. BCTF, Silver Spring, Maryland.

46. **Butinski, T. M.** 1988. Guenon birth seasons and correlates with rainfall and food, p. 284-322. *In* A. Gautier-Hion, F. Bourlière, J. P. Gautier, and J. Kingdon (ed.), A primate radiation: evolutionary biology of the African guenons. Cambridge University Press, Cambridge.

47. **Butynski, T. M.** 2001. Africa's Great Apes, p. 3-56. *In* B. Beck, T. S. Stoinski, M. Hutchins, T. L. Maple, B. Norton, A. Rowan, E. F. Stevens, and A. Arluke (ed.), Great Apes and Humans: The Ethics of Coexistence. Smithsonian Institution Press, Washington, D.C., USA.

48. **Butynski, T. M.** 1997. African Primate Conservation: The species and the IUCN/SSC Primate Specialist Group network. Primate Conservation **17:**87-100.

49. **Butynski, T. M., and J. Kalina.** 1998. Gorilla tourism: a critical look, p. 294-366. *In* E. J. Milner-Gulland and R. Mace (ed.), Conservation of biological resources. Blackwell Science, Oxford.

50. **Buzzard, P., and W. Eckardt.** 2007. The social system of the guenons, p. 51-71. *In* W. S. McGraw, K. Zuberbühler, and R. Noë (ed.), Monkeys of the Taï Forest. Cambridge University Press, Cambridge.

51. **Carr, J. K., J. N. Torimiro, N. D. Wolfe, M. N. Eitel, B. Kim, E. Sanders-Buell, L. L. Jagodzinski, D. Gotte, D. S. Burke, D. L. Birx, and F. E. McCutchan.** 2001. The AG recombinant IbNG and novel strains of group M HIV-1 are common in Cameroon. Virology **286:**168-81.

52. **Carter, J.** 2004. The Gambia, p. 51-54. *In* R. Kormos, C. Boesch, M. I. Bakarr, and T. M. Butinski (ed.), West African chimpanzees - Status Survey and Conservation Action Plan. IUCN publications, Cambridge, UK.

53. **Caspary, H. U.** 1999. Wildife utilisation in Côte d'Ivoire and West Africa-Potentials and constraints for development cooperation.

54. **Caspary, H. U., I. Koné, C. Prouot, and M. De Pauw.** 2001. La chasse et la filière viande de brousse dans l'espace Taï, Côte-d'Ivoire. Tropenbos Côte-d'Ivoire, p. 170 p, Séries 2. Tropenbos Côte-d'Ivoire, Abidjan.

55. **Caspary, H. U., and J. Momo.** 1998. La chasse villageoise en Côte d'Ivoire-résultats dans le cadre de l'étude Filière de viande de brousse (Enquête chasseurs), Rapport DPN et Banque Mondiale, Abidjan, Côte d'Ivoire. Rapport DPN et Banque Mondiale, Abidjan, Côte d 'ivoire.

56. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature **328:**543-7.

57. **Chapman, C. A., M. J. Lawes, and H. A. C. Eeley.** 2006. What hope for African primate diversity? Afr. J. Ecol. **44:**116-133.

58. **Chapuisat, M.** 1996. Characterization of microsatellite loci in Formica lugubris B and their variability in other ant species. Molecular Ecology **5:**599-601.

59. **Charleston, M. A., and D. L. Robertson.** 2002. Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. Syst Biol **51:**528-35.

60. **Charneau, P., A. M. Borman, C. Quillent, D. Guetard, S. Chamaret, J. Cohen, G. Remy, L. Montagnier, and F. Clavel.** 1994. Isolation and envelope sequence of a highly divergent HIV-1 isolate: definition of a new HIV-1 group. Virology **205:**247-53.

61. **Chen, Z., A. Luckay, D. L. Sodora, P. Telfer, P. Reed, A. Gettie, J. M. Kanu, R. F. Sadek, J. Yee, D. D. Ho, L. Zhang, and P. A. Marx.** 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J Virol **71:**3953-60.

62. **Chen, Z., P. Telfer, A. Gettie, P. Reed, L. Zhang, D. D. Ho, and P. A. Marx.** 1996. Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. J Virol **70:**3617-27.

63. **Chen, Z., P. Telfer, P. Reed, L. Zhang, A. Getti, D. D. Ho, and P. A. Marx.** 1995. Isolation and characterization of the first simian immunodeficiency virus from a feral sooty mangabey (*Cercocebus atys*) in West Africa. Journal of Medical Primatology **24:**108-15.

64. **Chen, Z., P. Zhou, D. D. Ho, N. R. Landau, and P. A. Marx.** 1997. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J. Virol. **71:**2705-2714.

65. **Cheng-Mayer, C., R. Liu, N. R. Landau, and L. Stamatatos.** 1997. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. J Virol **71:**1657-61.

66. **Clavel, F., F. Brun-Vezinet, D. Guetard, S. Chamaret, A. Laurent, C. Rouzioux, M. Rey, C. Katlama, F. Rey, J. L. Champelinaud, and et al.** 1986. [LAV type II: a second retrovirus associated with AIDS in West Africa]. C R Acad Sci III **302:**485-8.

67. **Clewley, J. P., J. C. Lewis, D. W. Brown, and E. L. Gadsby.** 1998. A novel simian immunodeficiency virus (SIVdrl) pol sequence from the drill monkey, *Mandrillus leucophaeus*. J Virol **72:**10305-9.

68. **Clisson, I., M. Lathuilliere, and B. Crouau-Roy.** 2000. Conservation and evolution of microsatellite loci in primate taxa. Am J Primatol **50:**205-14.

69. **Coatney, G. R., W. E. Collins, M. Warren, and P. G. Contacos.** 1971. The primate malarias U.S. Government Printing Office, Washington.

70. **Coffin, J. M.** 1992. Genetic diversity and evolution of retroviruses. Curr Top Microbiol Immunol **176:**143-64.

71. **Cooper, R., A. Feistner, S. Evans, H. Tsujimoto, and M. Hayami.** 1989. A lack of evidence of sexual transmission of a simian immunodeficiency agent in a semifree-ranging group of mandrills. Aids **3:**764.

72. **Coote, T., and M. W. Bruford.** 1996. Human microsatellites applicable for analysis of genetic variation in apes and Old World monkeys. Journal of Heredity **87:**406-10.

73. **Corbet, S., M. C. Muller-Trutwin, P. Versmisse, S. Delarue, A. Ayouba, J. Lewis, S. Brunak, P. Martin, F. Brun-Vezinet, F. Simon, F. Barre-Sinoussi, and P. Mauclere.** 2000. env sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. J Virol **74:**529-34.

74. **Cords, M.** 1988. Mating system of forest guenons: a preliminary review. , p. 323-339. *In* A. Gautier-Hion, F. Bourlière, J. P. Gautier, and J. Kingdon (ed.), A primate radiation: evolutionary biology of the African guenons. Cambridge University Press, Cambridge.

75. **Cords, M.** 2000. The number of males in guenon groups, p. 84-96. *In* P. M. Kappeler (ed.), Primate Males: Causes and Consequences of Variation in Group Composition Cambridge University Press, Cambridge.

76. **Courgnaud, V., B. Abela, X. Pourrut, E. Mpoudi-Ngole, S. Loul, E. Delaporte, and M. Peeters.** 2003. Identification of a new simian immunodeficiency virus lineage with a vpu gene present among different cercopithecus monkeys (*C. mona*, *C. cephus*, and *C. nictitans*) from Cameroon. J Virol **77:**12523-34.

77. **Courgnaud, V., P. Formenty, C. Akoua-Koffi, R. Noe, C. Boesch, E. Delaporte, and M. Peeters.** 2003. Partial molecular characterization of two simian immunodeficiency viruses (SIV) from African colobids: SIVwrc from Western red colobus (Piliocolobus badius) and SIVolc from olive colobus (Procolobus verus). J Virol **77:**744-8.

78. **Courgnaud, V., X. Pourrut, F. Bibollet-Ruche, E. Mpoudi-Ngole, A. Bourgeois, E. Delaporte, and M. Peeters.** 2001. Characterization of a novel simian immunodeficiency virus

from guereza colobus monkeys (*Colobus guereza*) in Cameroon: a new lineage in the nonhuman primate lentivirus family. J Virol **75:**857-66.

79. **Courgnaud, V., M. Salemi, X. Pourrut, E. Mpoudi-Ngole, B. Abela, P. Auzel, F. Bibollet-Ruche, B. Hahn, A. M. Vandamme, E. Delaporte, and M. Peeters.** 2002. Characterization of a novel simian immunodeficiency virus with a vpu gene from greater spot-nosed monkeys (*Cercopithecus nictitans*) provides new insights into simian/human immunodeficiency virus phylogeny. J Virol **76:**8298-309.

80. **Cullen, B. R.** 2001. Journey to the center of the cell. Cell **105:**697-700.

81. **Damond, F., C. Apetrei, D. L. Robertson, S. Souquiere, A. Lepretre, S. Matheron, J. C. Plantier, F. Brun-Vezinet, and F. Simon.** 2001. Variability of human immunodeficiency virus type 2 (HIV-2) infecting patients living in France. Virology **280:**19-30.

82. **Damond, F., M. Worobey, P. Campa, I. Farfara, G. Colin, S. Matheron, F. Brun-Vezinet, D. L. Robertson, and F. Simon.** 2004. Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification. AIDS Res Hum Retroviruses **20:**666-72.

83. **Daniel, M. D., Y. Li, Y. M. Naidu, P. J. Durda, D. K. Schmidt, C. D. Troup, D. P. Silva, J. J. MacKey, H. W. Kestler, 3rd, P. K. Sehgal, and et al.** 1988. Simian immunodeficiency virus from African green monkeys. J Virol **62:**4123-8.

84. **Dasilva, G. L.** 1992. The western black and white colobus as a low energy strategist: activity budgets, energy expenditure and energy intake. J Anim Ecol **61:**79-91.

85. **Daszak, P.** 2006. Risky behaviour in the Ebola zone. Anim Conserv **9:**366-367.

86. **Davies, A. G., and J. F. Oates.** 1994. Colobine Monkeys: their ecology, behaviour and evolution. Cambridge University Press, Cambridge.

87. **Dazza, M. C., M. Ekwalanga, M. Nende, K. B. Shamamba, P. Bitshi, D. Paraskevis, and S. Saragosti.** 2005. Characterization of a novel vpu-harboring simian immunodeficiency virus from a Dent's Mona monkey (*Cercopithecus mona denti*). J Virol **79:**8560-71.

88. **Delson, E.** 1992. Evolution of Old World Monkeys, p. 217-222. *In* J. S. Jones, R. D. Martin, D. Pilbeam, and S. Bunney (ed.), Cambridge Encyclopedia of Human Evolution. Cambridge University Press, Cambridge.

89. **deMenocal, P. B.** 2004. African climate change and faunal evolution during the Pliocene-Pleistocene. Earth and Planetary Science Letters **220:**3-24.

90. **Dimmic, M. W., J. S. Rest, D. P. Mindell, and R. A. Goldstein.** 2002. rtREV: an amino acid substitution matrix for inference of retrovirus and reverse transcriptase phylogeny. J Mol Evol **55:**65-73.

91. **Diop, O. M., A. Gueye, A. Ayouba, E. Nerrienet, S. Corbet, P. Mauclere, F. Simon, F. Barre-Sinoussi, and M. C. Muller-Trutwin.** 2002. Simian immunodeficiency viruses and the origin of HIVs, 2nd ed. . Kluwer Academic/Plenum Publishers, Dordrecht, The Netherlands.

92. **Diop, O. M., A. Gueye, M. Dias-Tavares, C. Kornfeld, A. Faye, P. Ave, M. Huerre, S. Corbet, F. Barre-Sinoussi, and M. C. Muller-Trutwin.** 2000. High levels of viral replication during primary simian immunodeficiency virus SIVagm infection are rapidly and strongly controlled in African green monkeys. J Virol **74:**7538-47.

93. **Disotell, T. R.** 1996. The phylogeny of Old World Monkeys. Evol Anthropol **5:**18-24.

94. **Domingo, E., and J. J. Holland.** 1997. RNA virus mutations and fitness for survival. Annu Rev Microbiol **51:**151-178.

95. **Douek, D. C., M. R. Betts, B. J. Hill, S. J. Little, R. Lempicki, J. A. Metcalf, J. Casazza, C. Yoder, J. W. Adelsberger, R. A. Stevens, M. W. Baseler, P. Keiser, D. D. Richman, R. T. Davey, and R. A. Koup.** 2001. Evidence for increased T cell turnover and decreased thymic output in HIV infection. J Immunol **167:**6663-8.

96. **Draulans, D., and E. Van Krunkelsven.** 2002. The impact ofwar on forest areas in the Democratic Republic of Congo. Oryx **36:**35-40.

97. **Dudley, J. P., J. R. Ginsberg, A. J. Plumptre, J. A. Hart, and L. C. Campos.** 2002. Effects of war and civil strife on wildlife and wildlife habitats. Conservation Biology **16:**319-329.

98. **Eckardt, W., and K. Zuberbühler.** 2004. Cooperation and competition in two forest monkeys. Behavioral Ecology **15:**400- 411.

99. **Emau, P., H. M. McClure, M. Isahakia, J. G. Else, and P. N. Fultz.** 1991. Isolation from African Sykes' monkeys (*Cercopithecus mitis*) of a lentivirus related to human and simian immunodeficiency viruses. J Virol **65:**2135-40.

100. **Enstam, K. L., and L. A. Isbell.** 2007. The guenons (Genus Cercopithecus) and their allies, p. 252-274. *In* B. Campbell, A. Fuentes, C. K. MacKinnon, M. Panger, and S. K. Bearder (ed.), Primates In perspective. Oxford University Press, Oxford, UK.

101. **Estaquier, J., M. Peeters, L. Bedjabaga, C. Honore, P. Bussi, A. Dixson, and E. Delaporte.** 1991. Prevalence and transmission of simian immunodeficiency virus and simian T-cell leukemia virus in a semi-free-range breeding colony of mandrills in Gabon. Aids **5:**1385-6.

102. **Esteves, A., R. Parreira, J. Piedade, T. Venenno, and W. F. Canas-Ferreira.** 2000. Genetic characterization of HIV type 1 and type 2 from Bissau, Guinea- Bissau (West Africa). Virus Res **68:**51-61.

103. **Fa, J., J. Juste, J. del Val, and J. Castroviejo.** 1995. Impact of market hunting on mammal species in Equatorial Guinea. Conserv. Biol. **9:**1107-1115.

104. **Felsenstein, J.** 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol **17:**368-76.

105. **Ferguson, A., K. A. Humphreys, and N. M. Croft.** 1995. Technical report: results of immunological tests on faecal extracts are likely to be misleading. Clin Exp Immunol **99:**70-75.

106. **Fitzgibbon, C. D., H. Mogaka, and J. H. Fanshave.** 1995. Subsistence hunting in Arabuko-Sokoke Forest, Kenya, and its effects on mammal populations. Conserv. Biol. **9:**1116-1126.

107. **Fleagle, J. G.** 1999. Primate Adaptation and Evolution, Second ed. Academic Press, San Diego.

108. **Formenty, P., C. Boesch, M. Wyers, C. Steiner, F. Donati, F. Dind, F. Walker, and B. Le Guenno.** 1999. Ebola virus outbreak among wild chimpanzees living in a rain forest of Cote d'Ivoire. J Infect Dis **179 Suppl 1:**S120-6.

109. **Froland, S. S., P. Jenum, C. F. Lindboe, K. W. Wefring, P. J. Linnestad, and T. Bohmer.** 1988. HIV-1 infection in a Norwegian family before 1970. Lancet **8598:**1344-5.

110. **Fultz, P. N., D. C. Anderson, H. M. McClure, S. Dewhurst, and J. I. Mullins.** 1990. SIVsmm infection of macaque and mangabey monkeys: correlation between in vivo and in vitro properties of different isolates. Dev Biol Stand **72:**253-8.

111. **Fultz, P. N., T. P. Gordon, D. C. Anderson, and H. M. McClure.** 1990. Prevalence of natural infection with simian immunodeficiency virus and simian T-cell leukemia virus type I in a breeding colony of sooty mangabey monkeys. Aids **4:**619-25.

112. **Fultz, P. N., P. Nara, F. Barre-Sinoussi, A. Chaput, M. L. Greenberg, E. Muchmore, M. P. Kieny, and M. Girard.** 1992. Vaccine protection of chimpanzees against challenge with HIV-1-infected peripheral blood mononuclear cells. Science **256:**1687-90.

113. **Fultz, P. N., R. B. Stricker, H. M. McClure, D. C. Anderson, W. M. Switzer, and C. Horaist.** 1990. Humoral response to SIV/SMM infection in macaque and mangabey monkeys. J Acquir Immune Defic Syndr **3:**319-29.

114. **Gagneux, P.** 2002. The genus Pan: population genetics of an endangered outgroup. Trends Genet **18:**327-30.

115. **Gagneux, P., C. Boesch, and D. S. Woodruff.** 1997. Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. Molecular Ecology **6:**861-8.

116. **Gagneux, P., C. Wills, U. Gerloff, D. Tautz, P. A. Morin, C. Boesch, B. Fruth, G. Hohmann, O. A. Ryder, and D. S. Woodruff.** 1999. Mitochondrial sequences show diverse evolutionary histories of African hominoids. Proc Natl Acad Sci U S A **96:**5077-82.

117. **Galat-Luong, A.** 1988. Monkeys in the Pirang forest. In:Pirang. Ecological Investigations in a Forest Island in the Gambia. Stiftung Walderhaltung in Afrika, Hamburg, and Bundesforschungsanstalt für Forst- und Holzwirtschaft, Hamburg, Warnke Verlag, Reinbek.

118. **Galat-Luong, A., and G. Galat.** 2005. Conservation and Survival Adaptations of Temminck's Red Colobus (*Procolobus badius temmincki*), in Senegal. Int J Primatol **26:**585-603.

119. **Galat, G., and A. Galat-Luong.** 1985. La communauté de primates diurnes de la forêt de Taï, Côte d'Ivoire. Rev. Ecol. (Terre Vie)**:**3-32.

120. **Galtier, N., M. Gouy, and C. Gautier.** 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci **12:**543-8.

121. **Galvani, A. P.** 2004. Emerging infections: what have we learned from SARS? Emerg Infect Dis **10:**1351-2.

122. **Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. Nature **397:**436-41.

123. **Gao, F., L. Yue, D. L. Robertson, S. C. Hill, H. Hui, R. J. Biggar, A. E. Neequaye, T. M. Whelan, D. D. Ho, G. M. Shaw, and et al.** 1994. Genetic diversity of human

immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J Virol **68:**7433-47.

124. **Gao, F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw, and B. H. Hahn.** 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature **358:**495-9.

125. **Gardner, M. B.** 1996. The history of simian AIDS. J Med Primatol **25:**148-157

126. **Garrett, L.** 1995. The Coming Plague: Newly Emerging diseases in a World Out of Balance, Penguin Books ed.

127. **Georges-Courbot, M. C., C. Y. Lu, M. Makuwa, P. Telfer, R. Onanga, G. Dubreuil, Z. Chen, S. M. Smith, A. Georges, F. Gao, B. H. Hahn, and P. A. Marx.** 1998. Natural infection of a household pet red-capped mangabey (*Cercocebus torquatus torquatus*) with a new simian immunodeficiency virus. J Virol **72:**600-8.

128. **Georges-Courbot, M. C., P. Moisson, E. Leroy, A. M. Pingard, E. Nerrienet, G. Dubreuil, E. J. Wickings, F. Debels, I. Bedjabaga, V. Poaty-Mavoungou, N. T. Hahn, and A. J. Georges.** 1996. Occurrence and frequency of transmission of naturally occurring simian retroviral infections (SIV, STLV, and SRV) at the CIRMF Primate Center, Gabon. J Med Primatol **25:**313-26.

129. **Gifford, R., and M. Tristem.** 2003. The evolution, distribution and diversity of endogenous retroviruses. Virus Genes **26:**291-315.

130. **Girard, M., J. Mahoney, Q. Wei, E. van der Ryst, E. Muchmore, F. Barre-Sinoussi, and P. N. Fultz.** 1998. Genital infection of female chimpanzees with human immunodeficiency virus type 1. AIDS Res Hum Retroviruses **14:**1357-67.

131. **Goff, S. P.** 2004. HIV: replication trimmed back. Nature **427:**791-793.

132. **Goldstein, S., I. Ourmanov, C. R. Brown, R. Plishka, A. Buckler-White, R. Byrum, and V. M. Hirsch.** 2005. Plateau levels of viremia correlate with the degree of CD4+-T-cell loss in simian immunodeficiency virus SIVagm-infected pigtailed macaques: variable pathogenicity of natural SIVagm isolates. J Virol **79:**5153-62.

133. **Gonder, M.** 2000. Evolutionary genetics of chimpanzees (*Pan troglodytes*) in Nigeria and Cameroon. Ph.D Dissertation. CUNY., New York.

134. **Gonder, M. K., J. F. Oates, T. R. Disotell, M. R. Forstner, J. C. Morales, and D. J. Melnick.** 1997. A new west African chimpanzee subspecies? Nature **388:**337.

135. **Goossens, B., L. Chikhi, S. Utami, J. R. de Ruiter, and M. W. Bruford.** 2000. A multisamples, multi-extracts approach for microsatellite analysis of faecal samples in an arboreal ape. Conservation Genetics **1:**157-162.

136. **Goossens, B., S. Latour, C. Vidal, A. Jamart, M. Ancrenaz, and M. W. Bruford.** 2000. Twenty new microsatellite loci for use with hair and faecal samples in the chimpanzee (*Pan troglodytes troglodytes*). Folia Primatologica (Basel) **71:**177-80.

137. **Graczyk, T. K., A. B. Mudakikwa, M. R. Cranfield, and U. Eilenberger.** 2001. Hyperkeratotic mange caused by Sarcoptes scabiei (Acariformes: Sarcoptidae) in juvenile human-habituated mountain gorillas (*Gorilla gorilla beringei*). Parasitol Res **87:**1024-1028.

138. **Greene, W. C., and B. M. Peterlin.** 2002. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat Med **8:**673-80.

139. **Groves, C.** 2001. Primate Taxonomy. Smithsonian Institution Press, Washington.

140. **Grubb, P.** 1999. Evolutionary processes implicit in distribution patterns of modern African mammals. *In* T. G. Bromage and F. Schrenk (ed.), African biogeography, climate change & human evolution. Oxford University Press, New York.

141. **Grubb, P.** 1982. Refuges and dispersal in the speciation of African forest mammals, p. 537-553. *In* G. T. Prance (ed.), Biological Diversification in the Tropics. Columbia University Press, New York.

142. **Grubb, P., T. Butinsky, J. Oates, S. Bearder, T. Disotell, C. Groves, and T. Struhsaker.** 2003. Assessment of the diversity of African primates. Int J Primatol **24:**1301-1357.

143. **Hahn, B. H., G. M. Shaw, K. M. De Cock, and P. M. Sharp.** 2000. AIDS as a zoonosis: scientific and public health implications. Science **287:**607-14.

144. **Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger, and M. H. Malim.** 2003. DNA deamination mediates innate immunity to retroviral infection. Cell **113:**803-9.

145. **Hasegawa, M., H. Kishino, and T. Yano.** 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol **22:**160-74.

146. **Heeney, J. L., E. Rutjens, E. J. Verschoor, H. Niphuis, P. ten Haaft, S. Rouse, H. McClure, S. Balla-Jhagjhoorsingh, W. Bogers, M. Salas, K. Cobb, L. Kestens, D. Davis, G. van der Groen, V. Courgnaud, M. Peeters, and K. K. Murthy.** 2006. Transmission of simian immunodeficiency virus SIVcpz and the evolution of infection in the presence and absence of concurrent human immunodeficiency virus type 1 infection in chimpanzees. J Virol **80:**7208-18.

147. **Hemelaar, J., E. Gouws, P. D. Ghys, and S. Osmanov.** 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. Aids **20:**W13-23.

148. **Heneine, W., W. M. Switzer, P. Sandstrom, J. Brown, S. Vedapuri, C. A. Schable, A. S. Khan, N. W. Lerche, M. Schweizer, D. Neumann-Haefelin, L. E. Chapman, and T. M. Folks.** 1998. Identification of a human population infected with simian foamy viruses. Nat Med **4:**403-7.

149. **Hill, C. M.** 1994. The role of female Diana monkeys, *Cercopithecus diana*, in territorial defense. Animal Behaviour **47:**425-431.

150. **Hill, W. C. O.** 1969. The nomenclature, taxonomy and distribution of chimpanzees, p. 22-49. *In* G. H. Bourne (ed.), The Chimpanzee. Anatomy, Behaviour and Diseases of Chimpanzees, vol. 1. Karger, Basel, Switzerland.

151. **Hirsch, V. M., B. J. Campbell, E. Bailes, R. Goeken, C. Brown, W. R. Elkins, M. Axthelm, M. Murphey-Corb, and P. M. Sharp.** 1999. Characterization of a novel simian immunodeficiency virus (SIV) from L'Hoest monkeys (*Cercopithecus l'hoesti*): implications for the origins of SIVmnd and other primate lentiviruses. J Virol **73:**1036-45.

152. **Hirsch, V. M., G. Dapolito, C. McGann, R. A. Olmsted, R. H. Purcell, and P. R. Johnson.** 1989. Molecular cloning of SIV from sooty mangabey monkeys. J Med Primatol **18:**279-85.

153. **Hirsch, V. M., G. A. Dapolito, S. Goldstein, H. McClure, P. Emau, P. N. Fultz, M. Isahakia, R. Lenroot, G. Myers, and P. R. Johnson.** 1993. A distinct African lentivirus from Sykes' monkeys. J Virol **67:**1517-28.

154. **Hirsch, V. M., C. McGann, G. Dapolito, S. Goldstein, A. Ogen-Odoi, B. Biryawaho, T. Lakwo, and P. R. Johnson.** 1993. Identification of a new subgroup of SIVagm in tantalus monkeys. Virology **197:**426-30.

155. **Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson.** 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature **339:**389-92.

156. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123-6.

157. **Hoelscher, M., W. E. Dowling, E. Sanders-Buell, J. K. Carr, M. E. Harris, A. Thomschke, M. L. Robb, D. L. Birx, and F. E. McCutchan.** 2002. Detection of HIV-1 subtypes, recombinants, and dual infections in east Africa by a multi-region hybridization assay. Aids **16:**2055-64.

158. **Holmes, E. C.** 2003. Molecular clocks and the puzzle of RNA virus origins. J Virol **77:**3893-7.

159. **Hooper, E.** 2001. Experimental oral polio vaccines and acquired immune deficiency syndrome. Philos Trans R Soc Lond B Biol Sci **356:**803-14.

160. **Hooper, E.** 1999. The river. A journey to the source of HIV and AIDS. Little, Brown and Company, Boston, USA.

161. **Hoppe-Dominik, B.** 1984. Etude du spectre des proies de la panthère, *Panthera pardus*, dans le Parc National de Taï en Côte d'Ivoire. Mammalia **48:**477-487.

162. **Hu, J., W. M. Switzer, B. T. Foley, D. L. Robertson, R. M. Goeken, B. T. Korber, V. M. Hirsch, and B. E. Beer.** 2003. Characterization and comparison of recombinant simian immunodeficiency virus from drill (*Mandrillus leucophaeus*) and mandrill (*Mandrillus sphinx*) isolates. J Virol **77:**4867-80.

163. **Hu, W. S., and H. M. Temin.** 1990. Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. Proc Natl Acad Sci U S A **87:**1556-60.

164. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754-5.

165. **Huet, T., R. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson.** 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature **345:**356-9.

166. **Huff, J. L., and P. A. Barry.** 2003. B-virus (Cercopithecine herpesvirus 1) infection in humans and macaques: potential for zoonotic disease. Emerg Infect Dis **9:**246-50.

167. **Hull, D. B.** 1979. A craniometric study of the black and white Colobus Illiger 1811 (Primates: Ceropithecoidea). Am J Phys Anthropol **51:**163-81.

168. **Jin, M. J., H. Hui, D. L. Robertson, M. C. Muller, F. Barre-Sinoussi, V. M. Hirsch, J. S. Allan, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1994. Mosaic genome structure of simian immunodeficiency virus from west African green monkeys. Embo J **13:**2935-47.

169. **Jin, M. J., J. Rogers, J. E. Phillips-Conroy, J. S. Allan, R. C. Desrosiers, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1994. Infection of a yellow baboon with simian immunodeficiency virus from African green monkeys: evidence for cross-species transmission in the wild. J Virol **68:**8454-60.

170. **Joag, S. V., Z. Li, L. Foresman, D. M. Pinson, R. Raghavan, W. Zhuge, I. Adany, C. Wang, F. Jia, D. Sheffer, J. Ranchalis, A. Watson, and O. Narayan.** 1997. Characterization of the pathogenic KU-SHIV model of acquired immunodeficiency syndrome in macaques. AIDS Res Hum Retroviruses **13:**635-45.

171. **Joag, S. V., Z. Li, L. Foresman, E. B. Stephens, L. J. Zhao, I. Adany, D. M. Pinson, H. M. McClure, and O. Narayan.** 1996. Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. J Virol **70:**3189-97.

172. **Johnson, P. R., S. Goldstein, W. T. London, A. Fomsgaard, and V. M. Hirsch.** 1990. Molecular clones of SIVsm and SIVagm: experimental infection of macaques and African green monkeys. J Med Primatol **19:**279-86.

173. **Jones-Engel, L., G. A. Engel, J. Heidrich, M. Chalise, N. Poudel, R. Viscidi, P. A. Barry, J. S. Allan, R. Grant, and R. Kyes.** 2006. Temple monkeys and health implications of commensalism, Kathmandu, Nepal. Emerg Infect Dis **12:**900-6.

174. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci **8:**275-82.

175. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21-132. *In* H. N. Munro (ed.), Mammalian Protein Metabolism. Academic Press, New York.

176. **Kaiser, S. M., H. S. Malik, and M. Emerman.** 2007. Restriction of an extinct retrovirus by the human TRIM5alpha antiviral protein. Science **316:**1756-8.

177. **Kalish, M. L., K. E. Robbins, D. Pieniazek, A. Schaefer, N. Nzilambi, T. C. Quinn, M. E. St Louis, A. S. Youngpairoj, J. Phillips, H. W. Jaffe, and T. M. Folks.** 2004. Recombinant viruses and early global HIV-1 epidemic. Emerg Infect Dis **10:**1227-34.

178. **Kalish, M. L., N. D. Wolfe, C. B. Ndongmo, J. McNicholl, K. E. Robbins, M. Aidoo, P. N. Fonjungo, G. A. Alemnji, C. Zeh, C. F. Djoko, E. Mpoudi-Ngole, D. S. Burke, and T. M. Folks.** 2005. Central African hunters exposed to simian immunodeficiency virus. Emerg Infect Dis **11:**1928-30.

179. **Kalter, S. S., C. H. Millstein, L. H. Boncyk, and L. B. Cummins.** 1978. Tuberculosis in nonhuman primates as a threat to humans. Dev Biol Stand. **41:**85-91.

180. **Kaur, A., L. Alexander, S. I. Staprans, L. Denekamp, C. L. Hale, H. M. McClure, M. B. Feinberg, R. C. Desrosiers, and R. P. Johnson.** 2001. Emergence of cytotoxic T lymphocyte escape mutations in nonpathogenic simian immunodeficiency virus infection. Eur J Immunol **31:**3207-17.

181. **Kaur, A., R. M. Grant, R. E. Means, H. McClure, M. Feinberg, and R. P. Johnson.** 1998. Diverse host responses and outcomes following simian immunodeficiency virus SIVmac239 infection in sooty mangabeys and rhesus macaques. J Virol **72:**9597-611.

182. **Kay, R. N. B., and A. G. Davies.** 1994. Digestive physiology, p. 229-249. *In* A. G. Davies and J. F. Oates (ed.), Colobine monkeys. Their Ecology, Behaviour and Evolution. Cambridge University Press, Cambridge.

183. **Keele, B. F., F. Van Heuverswyn, Y. Li, E. Bailes, J. Takehisa, M. L. Santiago, F. Bibollet-Ruche, Y. Chen, L. V. Wain, F. Liegeois, S. Loul, E. M. Ngole, Y. Bienvenue, E. Delaporte, J. F. Brookfield, P. M. Sharp, G. M. Shaw, M. Peeters, and B. H. Hahn.** 2006. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science **313:**523-6.

184. **Khabbaz, R. F., W. Heneine, J. R. George, B. Parekh, T. Rowe, T. Woods, W. M. Switzer, H. M. McClure, M. Murphey-Corb, and T. M. Folks.** 1994. Brief report: infection of a laboratory worker with simian immunodeficiency virus. N Engl J Med **330:**172-7.

185. **Kientz, A.** 1992. Protection du Parc National de Taï et gestion des terroirs par la population riveraine. Rapport établi par ordre et pour le compte de GTZ.

186. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16:**111-20.

187. **Kingdon, J.** 1997. The Kingdon field Guide to African Mammals. Academic Press, London.

188. **Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B. H. Hahn, S. Wolinsky, and T. Bhattacharya.** 2000. Timing the ancestor of the HIV-1 pandemic strains. Science **288:**1789-96.

189. **Kormos, R., M. I. Bakarr, L. Bonnéhin, and R. Hanson-Alp.** 2004. La chasse pour la viande de brousse et les chimpanzés en Afrique de l'Ouest. IUCN-Primates specialist group, Gland, Suisse.

190. **Kornfeld, C., M. J. Ploquin, I. Pandrea, A. Faye, R. Onanga, C. Apetrei, V. Poaty-Mavoungou, P. Rouquet, J. Estaquier, L. Mortara, J. F. Desoutter, C. Butor, R. Le Grand, P. Roques, F. Simon, F. Barre-Sinoussi, O. M. Diop, and M. C. Muller-Trutwin.** 2005. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. J Clin Invest **115:**1082-91.

191. **Korstjens, A. H.** 2001. The mob, the secret sorority, and the phantoms. An analysis of the socio-ecological strategies of the three colobines of Taï. PhD Thesis. Utrecht University, Utrecht.

192. **Korstjens, A. H., K. Bergmann, C. Deffernez, M. Krebs, E. C. Nijssen, B. A. M. van Oirschot, C. Paukert, and E. P. Schippers.** 2007. How small-scale differences in food competition lead to different social systems in three colsely related sympatric colobines, p. 72-108. *In* W. S. McGraw, K. Zuberbühler, and R. Noë (ed.), Monkeys of the Taï Forest. Cambridge University Press, Cambridge.

193. **Korstjens, A. H., E. C. Nijssen, and R. Noë.** 2005. Inter-group relationships in western black-and-white colobus, *Colobus polykomos polykomos*. International Journal of Primatology **26:**1267-1289.

194. **Korstjens, A. H., and R. Noe.** 2004. Mating system of an exceptional primate, the olive colobus (*Procolobus verus*). Am J Primatol **62:**261-73.

195. **Korstjens, A. H., and E. P. Schippers.** 2003. Dispersal patterns among olive colobus in Taï National Park. International Journal of Primatology **62:**261-273.

196. **Korstjens, A. H., E. H. M. Sterck, and R. Noë.** 2002. How adaptive or phylogenetically inert is primate social behaviour? A Test with two sympatric colobines. Behaviour**:**203-225.

197. **Kraft, Z., N. R. Derby, R. A. McCaffrey, R. Niec, W. M. Blay, N. L. Haigwood, E. Moysi, C. J. Saunders, T. Wrin, C. J. Petropoulos, M. J. McElrath, and L. Stamatatos.** 2007. Macaques infected with a CCR5-tropic simian/human immunodeficiency virus (SHIV) develop broadly reactive anti-HIV neutralizing antibodies. J Virol **81:**6402-11.

198. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform **5:**150-63.

199. **Kuznetsov, Y. G., J. G. Victoria, W. E. Robinson, Jr., and A. McPherson.** 2003. Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes. J Virol **77:**11896-909.

200. **Lahm, S. A.** 1993. Utilization of forest resources and local variation of wildlife populations in Northeastern Gabon. *In* C. M. Hladik, A. Hladik, O. F. Linarea, H. Pagezy, A. Semple, and M. Hadley (ed.), Tropical Forest, People, and Food. Parthenon Publishing Group, Paris.

201. **Lanave, C., G. Preparata, C. Saccone, and G. Serio.** 1984. A new method for calculating evolutionary substitution rates. J Mol Evol **20:**86-93.

202. **Laporte, N. T., J. A. Stabach, R. Grosch, T. S. Lin, and S. J. Goetz.** 2007. Expansion of industrial logging in Central Africa. Science **316:**1451.

203. **Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon.** 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell **50:**975-85.

204. **Laurance, W. F.** 2001. Immense logging deal to sustain war in Congo. Trends in Ecology & Evolution **16:**670.

205. **Laurent, C., A. Bourgeois, M. Mpoudi, C. Butel, M. Peeters, E. Mpoudi-Ngole, and E. Delaporte.** 2004. Commercial logging and HIV epidemic, rural Equatorial Africa. Emerg Infect Dis **10:**1953-6.

206. **Leakey, M.** 1988. Fossil evidence for the evolution of the guenons, p. 7-12. *In* A. Gautier-Hion, F. Boulière, J. P. Gautier, and J. Kingdon (ed.), A primate radiation: Evolutionary biology of the African guenons.

207. **Leakey, M. G.** 2007. Cercopithecid assemblages in the Koobi Fora Formation, Omo-Turkana Basin, northern Kenya. American Journal of Physical Anthropology **Supplement 44:**152-153.

208. **Leendertz, F. H., C. Boesch, S. Junglen, G. Pauli, and H. Ellerbrok.** 2003. Characterization of a new simian T-lymphocyte virus type 1 (STLV-1) in a wild living chimpanzee (Pan troglodytes verus) from Ivory Coast: evidence of a new STLV-1 group? AIDS Res Hum Retroviruses **19:**255-8.

209. **Leendertz, F. H., S. Junglen, C. Boesch, P. Formenty, E. Couacy-Hymann, V. Courgnaud, G. Pauli, and H. Ellerbrok.** 2004. High variety of different simian T-cell leukemia virus type 1 strains in chimpanzees (*Pan troglodytes verus*) of the Tai National Park, Cote d'Ivoire. J Virol **78:**4352-6.

210. **Lehman, D. A., and C. Farquhar.** 2007. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. Rev Med Virol **17:**381-403.

211. **Lemey, P., O. G. Pybus, A. Rambaut, A. J. Drummond, D. L. Robertson, P. Roques, M. Worobey, and A. M. Vandamme.** 2004. The molecular population genetics of HIV-1 group O. Genetics **167:**1059-68.

212. **Lemey, P., O. G. Pybus, B. Wang, N. K. Saksena, M. Salemi, and A. M. Vandamme.** 2003. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci U S A **100:**6588-92.

213. **Levy, J. A.** 2007. HIV and the pathogenesis of AIDS. 3 ed. ASM Press, Washington DC, USA.

214. **Levy, J. A.** 2007. Laboratory diagnosis of acute and primary HIV infection, p. 81-82. *In* J. A. Levy (ed.), HIV and the pathogenesis of AIDS. 3 ed. ASM Press, Washington DC, USA.

215. **Liegeois, F., V. Courgnaud, W. M. Switzer, H. W. Murphy, S. Loul, A. Aghokeng, X. Pourrut, E. Mpoudi-Ngole, E. Delaporte, and M. Peeters.** 2006. Molecular characterization of a novel simian immunodeficiency virus lineage (SIVtal) from northern talapoins (Miopithecus ogouensis). Virology **349:**55-65.

216. **Ling, B., C. Apetrei, I. Pandrea, R. S. Veazey, A. A. Lackner, B. Gormus, and P. A. Marx.** 2004. Classic AIDS in a sooty mangabey after an 18-year natural infection. J Virol **78:**8902-8.

217. **Ling, B., M. L. Santiago, S. Meleth, B. Gormus, H. M. McClure, C. Apetrei, B. H. Hahn, and P. A. Marx.** 2003. Noninvasive detection of new simian immunodeficiency virus lineages in captive sooty mangabeys: ability to amplify virion RNA from fecal samples correlates with viral load in plasma. J Virol **77:**2214-26.

218. **Locatelli, S., B. Lafay, F. Liegeois, N. Ting, E. Delaporte, and M. Peeters.** 2008. Full molecular characterisation of a simian immunodeficiency virus, SIVwrc-pbt from Temminck's red colobus (*Piliocolobus badius temminckii*) from Abuko Nature Reserve, The Gambia. Virology, In press.

219. **Locatelli, S., F. Liegeois, B. Lafay, A. D. Roeder, M. W. Bruford, P. Formenty, R. Noë, E. Delaporte, and M. Peeters.** 2008. Prevalence and genetic diversity of Simian Immunodeficiency virus infection in wild-living red colobus monkeys (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire. Infection Genetics and Evolution **8:**1-14.

220. **Locatelli, S., F. Liegeois, B. Lafay, A. D. Roeder, M. W. Bruford, P. Formenty, R. Noë, E. Delaporte, and M. Peeters.** 2008. Prevalence and genetic diversity of Simian Immunodeficiency virus infection in wild-living red colobus monkeys (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire. Infect Genet Evol **8:**1-14.

221. **Lole, K. S., R. C. Bollinger, S. Ramesh, R. S. Paranjape, D. Gadkari, S. S. Kulkarni, N. G. Novak, R. ngersoll, H. W. Sheppard, and S. C. Ray.** 1999. Full-Length Human Immunodeficiency Virus Type 1 Genomes from Subtype C-Infected Seroconverters in India, with Evidence of Intersubtype Recombination. J Virol **73:**152-160.

222. **Lowenstine, L. J., N. W. Lerche, J. L. Yee, A. Uyeda, M. B. Jennings, R. J. Munn, H. M. McClure, D. C. Anderson, P. N. Fultz, and M. B. Gardner.** 1992. Evidence for a lentiviral etiology in an epizootic of immune deficiency and lymphoma in stump-tailed macaques (*Macaca arctoides*). J Med Primatol **21:**1-14.

223. **Lowenstine, L. J., N. C. Pedersen, J. Higgins, K. C. Pallis, A. Uyeda, P. Marx, N. W. Lerche, R. J. Munn, and M. B. Gardner.** 1986. Seroepidemiologic survey of captive Old-World primates for antibodies to human and simian retroviruses, and isolation of a lentivirus from sooty mangabeys (*Cercocebus atys*). Int J Cancer **38:**563-74.

224. **Luban, J., K. L. Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067-78.

225. **Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono.** 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **424:**99-103.

226. **Mansfield, K. G., N. W. Lerch, M. B. Gardner, and A. A. Lackner.** 1995. Origins of simian immunodeficiency virus infection in macaques at the New England Regional Primate Research Center. J Med Primatol **24:**116-22.

227. **Mansky, L. M., and H. M. Temin.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol **69:**5087-94.

228. **Marler, P.** 1969. Colobus guereza: territoriality and group composition. Science **163:**93-95.

229. **Martin-Serrano, J., T. Zang, and P. D. Bieniasz.** 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress Nat Med **7:**1313-9.

230. **Martin, C.** 1991. The Rainforests of West Africa: Ecology, Threaths, Conservation. Birkhäuser, Basel.

231. **Martin, G.** 1983. Bushmeat in Nigeria as a natural resource with environmental implications. Environ. Conserv. **10:**125-132.

232. **Marx, P. A., Y. Li, N. W. Lerche, S. Sutjipto, A. Gettie, J. A. Yee, B. H. Brotman, A. M. Prince, A. Hanson, R. G. Webster, and et al.** 1991. Isolation of a simian immunodeficiency virus related to human immunodeficiency virus type 2 from a west African pet sooty mangabey. J Virol **65:**4480-5.

233. **Masciotra, S., D. L. Rudolph, G. van der Groen, C. Yang, and R. B. Lal.** 2000. Serological detection of infection with diverse human and simian immunodeficiency viruses using consensus env peptides. Clin Diagn Lab Immunol **7:**706-9.

234. **McCutchan, F. E.** 2006. Global epidemiology of HIV. J Med Virol **78 Suppl 1:**S7-S12.

235. **McGraw, W. S.** 1996. Cercopithecid locomotion, support use, and support availability in the Tai Forest, Ivory Coast. Am J Phys Anthropol **100:**507-22.

236. **McGraw, W. S.** 2005. Update on the Search for Miss Waldron's Red Colobus Monkey Int J Primatol **26**.

237. **McGraw, W. S.** 2007. Vulnerability and conservation of the Taï monkey fauna, p. 290-316. *In* W. S. McGraw, K. Zuberbühler, and R. Noë (ed.), Monkeys of the Taï Forest. Cambridge University Press, Cambridge.

238. **McGraw, W. S., I. T. Monah, and M. Abedi-Lartey.** 1999. Survey of endangered primates in the forest reserves of eastern Côte d'Ivoire. African Primates **3:**22-25.

239. **McGraw, W. S., and K. Zuberbühler.** 2007. The Monkeys of the Taï forets: an introduction, p. 1-48. *In* W. S. McGraw, K. Zuberbühler, and R. Noë (ed.), Monkeys of the Taï forest. An African Primate community. Cambridge University Press, Cambridge.

240. **McGraw, W. S., K. Zuberbühler, and R. Noë.** 2007. Monkeys of the Taï Forest. An African Primate Community. Cambridge University Press, Cambridge.

241. **Miller, R. J., J. S. Cairns, S. Bridges, and N. Sarver.** 2000. Human immunodeficiency virus and AIDS: insights from animal lentiviruses. J Virol **74:**7187-95.

242. **Mitani, J. C., and D. P. Watts.** 1999. Demographic influences on the hunting behavior of chimpanzees. Am J Phys Anthropol **109:**439-54.

243. **Monroe, M. C., and A. S. Willcox.** 2006. Could risk of disease change bushmeat-butchering behavior? Anim Conserv **9:**368-369.

244. **Moore, J. P., S. G. Kitchen, P. Pugach, and J. A. Zack.** 2004. The CCR5 and CXCR4 coreceptors-central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. . AIDS Res.Hum. Retrovir. **20:**111-126.

245. **Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau.** 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science **250:**1139-42.

246. **Morens, D. M., G. K. Folkers, and A. S. Fauci.** 2004. The challenge of emerging and re-emerging infectious diseases. Nature **430:**242-9.

247. **Morin, P. A., J. J. Moore, R. Chakraborty, L. Jin, J. Goodall, and D. S. Woodruff.** 1994. Kin selection, social structure, gene flow, and the evolution of chimpanzees. Science **265:**1193-201.

248. **Morse, S. S.** 1995. Factors in the Emergence of Infectious Diseases. Emerg Infect Dis **1:**7-15.

249. **Murphey-Corb, M., L. N. Martin, S. R. Rangan, G. B. Baskin, B. J. Gormus, R. H. Wolf, W. A. Andes, M. West, and R. C. Montelaro.** 1986. Isolation of an HTLV-III-related retrovirus from macaques with simian AIDS and its possible origin in asymptomatic mangabeys. Nature **321:**435-7.

250. **Murray, S. M., and M. L. Linial.** 2006. Foamy virus infection in primates. J Med Primatol **35:**225-35.

251. **Murray, S. M., and M. L. Linial.** 2006. Foamy virus infection in primates. J Med Primatol **35:**225-235.

252. **Murthy, K. K., E. K. Cobb, S. R. Rouse, H. M. McClure, J. S. Payne, M. T. Salas, and G. R. Michalek.** 1998. Active and passive immunization against HIV type 1 infection in chimpanzees AIDS Res Hum Retroviruses **14** S271-6.

253. **Navidi, W., N. Arnheim, and M. S. Waterman.** 1992. A multiple-tubes approach for accurate genotyping of very small DNA samples by using PCR: Statistical considerations. American Journal of Human Genetics **50:**347-359.

254. **Ndongmo, C. B., W. M. Switzer, C. P. Pau, C. Zeh, A. Schaefer, D. Pieniazek, T. M. Folks, and M. L. Kalish.** 2004. New multiple antigenic peptide-based enzyme immunoassay for detection of simian immunodeficiency virus infection in nonhuman primates and humans. J Clin Microbiol **42:**5161-9.

255. **Nelson, G. S.** 1960. Schistosome infections as zoonoses in Africa. Trans R Soc Trop Med Hyg **54:**301-24.

256. **Nerrienet, E., X. Amouretti, M. C. Muller-Trutwin, V. Poaty-Mavoungou, I. Bedjebaga, H. T. Nguyen, G. Dubreuil, S. Corbet, E. J. Wickings, F. Barre-Sinoussi, A. J. Georges, and M. C. Georges-Courbot.** 1998. Phylogenetic analysis of SIV and STLV type I in mandrills (*Mandrillus sphinx*): indications that intracolony transmissions are predominantly the result of male-to-male aggressive contacts. AIDS Res Hum Retroviruses **14:**785-96.

257. **Nerrienet, E., M. L. Santiago, Y. Foupouapouognigni, E. Bailes, N. I. Mundy, B. Njinku, A. Kfutwah, M. C. Muller-Trutwin, F. Barre-Sinoussi, G. M. Shaw, P. M. Sharp, B. H. Hahn, and A. Ayouba.** 2005. Simian immunodeficiency virus infection in wild-caught chimpanzees from cameroon. J Virol **79:**1312-9.

258. **NIAID** 2004, posting date. How HIV causes AIDS. http://www.niaid.nih.gov/. [Online.]

259. **Noë, R., and R. Bshary.** 1997. The formation of red colobus - diana monkey associations under predation pressure from chimpanzees. Proc.R.Soc.B. **264:**251-253.

260. **North, T. W., K. K. Van Rompay, J. Higgins, T. B. Matthews, D. A. Wadford, N. C. Pedersen, and R. F. Schinazi.** 2005. Suppression of virus load by highly active antiretroviral therapy in rhesus macaques infected with a recombinant simian immunodeficiency virus

containing reverse transcriptase from human immunodeficiency virus type 1. J Virol **79:**7349-54.

261. **Novembre, F. J., J. de Rosayro, S. Nidtha, S. P. O'Neil, T. R. Gibson, T. Evans-Strickfaden, C. E. Hart, and H. M. McClure.** 2001. Rapid CD4(+) T-cell loss induced by human immunodeficiency virus type 1(NC) in uninfected and previously infected chimpanzees. J Virol **75:**1533-9.

262. **Novembre, F. J., M. Saucier, D. C. Anderson, S. A. Klumpp, S. P. O'Neil, C. R. Brown, 2nd, C. E. Hart, P. C. Guenthner, R. B. Swenson, and H. M. McClure.** 1997. Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol **71:**4086-91.

263. **Nunn, C. L.** 2002. A comparative study of leukocyte counts and disease risk in primates. Evolution Int J Org Evolution **56:**177-90.

264. **Nunn, C. L., and S. Altizer.** 2006. Infectious Diseases in Primates. Behavior, Ecology and Evolution. Oxford University Press, Oxford, UK.

265. **Nunn, C. L., S. Altizer, K. E. Jones, and W. Sechrest.** 2003. Comparative tests of parasite species richness in primates. Am Nat **162:**597-614.

266. **Nunn, C. L., J. L. Gittleman, and J. Antonovics.** 2000. Promiscuity and the primate immune system. Science **290:**1168-70.

267. **Nygren, A., T. Bergman, T. Matthews, H. Jornvall, and H. Wigzell.** 1988. 95- and 25-kDa fragments of the human immunodeficiency virus envelope glycoprotein gp120 bind to the CD4 receptor. Proc Natl Acad Sci U S A **85:**6543-6.

268. **O'Neil, S. P., F. J. Novembre, A. B. Hill, C. Suwyn, C. E. Hart, T. Evans-Strickfaden, D. C. Anderson, J. deRosayro, J. G. Herndon, M. Saucier, and H. M. McClure.** 2000. Progressive infection in a subset of HIV-1-positive chimpanzees. J Infect Dis **182:**1051-62.

269. **Oates, J. F.** 1994. The natural history of African colobines, p. 75-128. *In* A. G. Davies and J. F. Oates (ed.), Colobine monkeys. Cambridge University Press, Cambridge.

270. **Oates, J. F., M. Abedi-Lartey, W. S. McGraw, T. T. Struhsaker, and G. H. Whitesides.** 2000. Extinction of West African red colobus monkey. Conserv. Biol. **14:**1526-1532.

271. **Oates, J. F., C. M. Bocian, and C. J. Terranova.** 2000. Loud calls of black and white colobus monkeys: their adaptative and taxonomic significance in light of new data, p. 431-457. *In* P. W. Whitehead and C. J. Jolly (ed.), Old World Monkeys. Cambridge University press, Cambridge.

272. **Oates, J. F., A. G. Davies, and E. Delson.** 1994. The diversity of the living colobines, p. 45-74. *In* A. G. Davies and J. F. Oates (ed.), Colobine Monkeys: their ecology, behaviour, and evolution. Cambridge University Press, Cambridge.

273. **Oates, J. F., and T. F. Trocco.** 1983. Taxonomy and phylogeny of black-and-white colobus monkeys. Inferences from an analysis of loud call variation. Folia Primatol (Basel) **40:**83-113.

274. **Oates, J. F., and G. H. Whitesides.** 1990. Association between olive colobus (*Procolobus verus*), Diana guenons (*Cercopithecus diana*), and other forest monkeys in Sierra Leone. American Journal of Primatology **21:**129-146.

275. **Ohta, Y., T. Masuda, H. Tsujimoto, K. Ishikawa, T. Kodama, S. Morikawa, M. Nakai, S. Honjo, and M. Hayami.** 1988. Isolation of simian immunodeficiency virus from African green monkeys and seroepidemiologic survey of the virus in various non-human primates. Int J Cancer **41:**115-22.

276. **Onanga, R., S. Souquiere, M. Makuwa, A. Mouinga-Ondeme, F. Simon, C. Apetrei, and P. Roques.** 2006. Primary simian immunodeficiency virus SIVmnd-2 infection in mandrills (*Mandrillus sphinx*). J Virol **80:**3301-9.

277. **Osterhaus, A. D., N. Pedersen, G. van Amerongen, M. T. Frankenhuis, M. Marthas, E. Reay, T. M. Rose, J. Pamungkas, and M. L. Bosch.** 1999. Isolation and partial characterization of a lentivirus from talapoin monkeys (*Miopithecus talapoin*). Virology **260:**116-24.

278. **Otsyula, M., J. Yee, M. Jennings, M. Suleman, A. Gettie, R. Tarara, M. Isahakia, P. Marx, and N. Lerche.** 1996. Prevalence of antibodies against simian immunodeficiency virus (SIV) and simian T-lymphotropic virus (STLV) in a colony of non-human primates in Kenya, East Africa. Ann Trop Med Parasitol **90:**65-70.

279. **Owen, S. M., S. Masciotra, F. Novembre, J. Yee, W. M. Switzer, M. Ostyula, and R. B. Lal.** 2000. Simian immunodeficiency viruses of diverse origin can use CXCR4 as a coreceptor for entry into human cells. J Virol **74:**5702-8.

280. **PACPNT.** 1997. Rapport d'évaluation des activités du PACPNT d'octobre 96 à juillet 97.

281. **Pandrea, I., C. Apetrei, S. Gordon, J. Barbercheck, J. Dufour, R. Bohm, B. Sumpter, P. Roques, P. A. Marx, V. M. Hirsch, A. Kaur, A. A. Lackner, R. S. Veazey, and G. Silvestri.** 2007. Paucity of CD4+CCR5+ T cells is a typical feature of natural SIV hosts. Blood **109:**1069-76.

282. **Pandrea, I., C. Kornfeld, M. J. Ploquin, C. Apetrei, A. Faye, P. Rouquet, P. Roques, F. Simon, F. Barre-Sinoussi, M. C. Muller-Trutwin, and O. M. Diop.** 2005. Impact of viral factors on very early in vivo replication profiles in simian immunodeficiency virus SIVagm-infected African green monkeys. J Virol **79:**6249-59.

283. **Pandrea, I., R. Onanga, P. Rouquet, O. Bourry, P. Ngari, E. J. Wickings, P. Roques, and C. Apetrei.** 2001. Chronic SIV infection ultimately causes immunodeficiency in African non-human primates. Aids **15:**2461-2.

284. **Pandrea, I., G. Silvestri, R. Onanga, R. S. Veazey, P. A. Marx, V. Hirsch, and C. Apetrei.** 2006. Simian immunodeficiency viruses replication dynamics in African non-human primate hosts: common patterns and species-specific differences. J Med Primatol **35:**194-201.

285. **Pearce, F.** 2005. The protein gap. Conserv. Prac. **6:**21-27.

286. **Pedersen, A. B., S. Altizer, M. Poss, A. A. Cunningham, and C. L. Nunn.** 2005. Patterns of host specificity and transmission among parasites of wild primates. Int J Parasitol **35:**647-57.

287. **Peeters, M., V. Courgnaud, B. Abela, P. Auzel, X. Pourrut, F. Bibollet-Ruche, S. Loul, F. Liegeois, C. Butel, D. Koulagna, E. Mpoudi-Ngole, G. M. Shaw, B. H. Hahn, and E. Delaporte.** 2002. Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. Emerging Infectious Diseases **8:**451-7.

288. **Peeters, M., K. Fransen, E. Delaporte, M. Van den Haesevelde, G. M. Gershy-Damet, L. Kestens, G. van der Groen, and P. Piot.** 1992. Isolation and characterization of a new chimpanzee lentivirus (simian immunodeficiency virus isolate cpz-ant) from a wild-captured chimpanzee. AIDS **6:**447-51.

289. **Peeters, M., A. Gueye, S. Mboup, F. Bibollet-Ruche, E. Ekaza, C. Mulanga, R. Ouedrago, R. Gandji, P. Mpele, G. Dibanga, B. Koumare, M. Saidou, E. Esu-Williams, J. P. Lombart, W. Badombena, N. Luo, M. Vanden Haesevelde, and E. Delaporte.** 1997. Geographical distribution of HIV-1 group O viruses in Africa. Aids **11:**493-8.

290. **Peeters, M., C. Honore, T. Huet, L. Bedjabaga, S. Ossari, P. Bussi, R. W. Cooper, and E. Delaporte.** 1989. Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. AIDS **3:**625-30.

291. **Peeters, M., W. Janssens, K. Fransen, J. Brandful, L. Heyndrickx, K. Koffi, E. Delaporte, P. Piot, G. M. Gershy-Damet, and G. van der Groen.** 1994. Isolation of simian immunodeficiency viruses from two sooty mangabeys in Cote d'Ivoire: virological and genetic characterization and relationship to other HIV type 2 and SIVsm/mac strains. AIDS Res Hum Retroviruses **10:**1289-94.

292. **Peeters, M., F. Liegeois, N. Torimiro, A. Bourgeois, E. Mpoudi, L. Vergne, E. Saman, E. Delaporte, and S. Saragosti.** 1999. Characterization of a highly replicative intergroup M/O human immunodeficiency virus type 1 recombinant isolated from a Cameroonian patient. J Virol **73:**7368-75.

293. **Peeters, M., and P. M. Sharp.** 2000. Genetic diversity of HIV-1: the moving target. Aids **14 Suppl 3:**S129-40.

294. **Peters, I. R., E. L. Calvert, E. J. Hall, and M. J. Day.** 2004. Measurement of immunoglobulin concentrations in the feces of healthy dogs. Clin Diagn Lab Immunol **11:**841-8.

295. **Phillips-Conroy, J. E., C. J. Jolly, B. Petros, J. S. Allan, and R. C. Desrosiers.** 1994. Sexual transmission of SIVagm in wild grivet monkeys. J Med Primatol **23:**1-7.

296. **Pieniazek, D., D. Ellenberger, L. M. Janini, A. C. Ramos, J. Nkengasong, M. Sassan-Morokro, D. J. Hu, I. M. Coulibally, E. Ekpini, C. Bandea, A. Tanuri, A. E. Greenberg, S. Z. Wiktor, and M. A. Rayfield.** 1999. Predominance of human immunodeficiency virus type 2 subtype B in Abidjan, Ivory Coast. AIDS Res Hum Retroviruses **15:**603-8.

297. **Pitt, J., D. Brambilla, P. Reichelderfer, A. Landay, K. McIntosh, D. Burns, G. V. Hillyer, H. Mendez, and M. G. Fowler.** 1997. Maternal immunologic and virologic risk factors for infant human immunodeficiency virus type 1 infection: findings from the Women and Infants Transmission Study. J Infect Dis **175:**567-75.

298. **Plotkin, S. A.** 2001. CHAT oral polio vaccine was not the source of human immunodeficiency virus type 1 group M for humans. Clin Infect Dis **32:**1068-84.

299. **Plumptre, A. J., T. Hart, A. Vedder, and J. Robinson.** 2000. Support of Congolese conservationists. Science **288:**617.

300. **Poaty-Mavoungou, V., R. Onanga, I. Bedjabaga, and E. Mavoungou.** 2001. Simian immunodeficiency virus from mandrill (*Mandrillus sphinx*) SIVmnd experimentally infects human and nonhuman primate cells. Microbes Infect **3:**599-610.

301. **Poinar, H., M. Kuch, and S. Paabo.** 2001. Molecular analyses of oral polio vaccine samples. Science **292:**743-4.

302. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817-8.

303. **Prince, A. M., B. Brotman, D. H. Lee, L. Andrus, J. Valinsky, and P. Marx.** 2002. Lack of evidence for HIV type 1-related SIVcpz infection in captive and wild chimpanzees (*Pan troglodytes verus*) in West Africa. AIDS Res Hum Retroviruses **18:**657-60.

304. **Puffer, B. A., L. J. Parent, J. W. Wills, and R. C. Montelaro.** 1997. Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein J Virol **71:**6541-6.

305. **Raaum, R. L., K. N. Sterner, C. M. Noviello, C. B. Stewart, and T. R. Disotell.** 2005. Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence. J Hum Evol **48:**237-57.

306. **Range, F., and R. Noe.** 2002. Familiarity and dominance relations among female sooty mangabeys in the Tai National Park. Am J Primatol **56:**137-53.

307. **Rannala, B., and Z. Yang.** 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J Mol Evol **43:**304-311.

308. **Raymond, M., and F. Rousset.** 1995. Genepop (version 1.2), population genetics software for exact tests and ecumenicism. Journal of Heredity **86:**248-249.

309. **Reeves, J. D., and R. W. Doms.** 2002. Human immunodeficiency virus type 2. J Gen Virol **83:**1253-65.

310. **Refisch, J., and I. Koné.** 2005. Impact of commercial hunting on monkey populations in the Taï region, Côte d'Ivoire. Biotropica **37:**136-144.

311. **Renquist, D. M., and R. A. Whitney.** 1987. Zoonoses acquired from pet primates. Vet Clin North Am Small Anim Pract. **17:**219-40.

312. **Rey-Cuille, M. A., J. L. Berthier, M. C. Bomsel-Demontoy, Y. Chaduc, L. Montagnier, A. G. Hovanessian, and L. A. Chakrabarti.** 1998. Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. J Virol **72:**3872-86.

313. **Ronquist, F., and J. P. Huelsenbeck.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:**1572-4.

314. **Rothman, J., and D. D. Bowman.** 2003. A review of the endoparasites of mountain gorillas. *In* D. D. Bowman (ed.), Companion and Exotic Animal Parasitology. International Veterinary Information Service, Ithaca, NY.

315. **Ryder, R. W., W. Nsa, S. E. Hassig, F. Behets, M. Rayfield, B. Ekungola, A. M. Nelson, U. Mulenda, H. Francis, K. Mwandagalirwa, and et al.** 1989. Perinatal transmission of the human immunodeficiency virus type 1 to infants of seropositive women in Zaire. N Engl J Med **320:**1637-42.

316. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406-25.

317. **Sakuragi, S., R. Shibata, R. Mukai, T. Komatsu, M. Fukasawa, H. Sakai, J. Sakuragi, M. Kawamura, K. Ibuki, M. Hayami, and et al.** 1992. Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. J Gen Virol **73 ( Pt 11):**2983-7.

318. **Salemi, M., T. De Oliveira, V. Courgnaud, V. Moulton, B. Holland, S. Cassol, W. M. Switzer, and A. M. Vandamme.** 2003. Mosaic genomes of the six major primate lentivirus lineages revealed by phylogenetic analyses. J Virol **77:**7202-13.

319. **Salemi, M., K. Strimmer, W. W. Hall, M. Duffy, E. Delaporte, S. Mboup, M. Peeters, and A. M. Vandamme.** 2001. Dating the common ancestor of SIVcpz and HIV-1 group M and the origin of HIV-1 subtypes using a new method to uncover clock-like molecular evolution. Faseb J **15:**276-8.

320. **Santiago, M. L., F. Bibollet-Ruche, E. Bailes, S. Kamenya, M. N. Muller, M. Lukasik, A. E. Pusey, D. A. Collins, R. W. Wrangham, J. Goodall, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 2003. Amplification of a complete simian immunodeficiency virus genome from fecal RNA of a wild chimpanzee. Journal of Virology **77:**2233-42.

321. **Santiago, M. L., M. Lukasik, S. Kamenya, Y. Li, F. Bibollet-Ruche, E. Bailes, M. N. Muller, M. Emery, D. A. Goldenberg, J. S. Lwanga, A. Ayouba, E. Nerrienet, H. M. McClure, J. L.**

**Heeney, D. P. Watts, A. E. Pusey, D. A. Collins, R. W. Wrangham, J. Goodall, J. F. Brookfield, P. M. Sharp, G. M. Shaw, and B. H. Hahn.** 2003. Foci of endemic simian immunodeficiency virus infection in wild-living eastern chimpanzees (Pan troglodytes schweinfurthii). Journal of Virology **77:**7545-62.

322. **Santiago, M. L., F. Range, B. F. Keele, Y. Li, E. Bailes, F. Bibollet-Ruche, C. Fruteau, R. Noe, M. Peeters, J. F. Brookfield, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 2005. Simian immunodeficiency virus infection in free-ranging sooty mangabeys (*Cercocebus atys atys*) from the Tai Forest, Cote d'Ivoire: implications for the origin of epidemic human immunodeficiency virus type 2. Journal of Virology **79:**12515-27.

323. **Santiago, M. L., C. M. Rodenburg, S. Kamenya, F. Bibollet-Ruche, F. Gao, E. Bailes, S. Meleth, S. J. Soong, J. M. Kilby, Z. Moldoveanu, B. Fahey, M. N. Muller, A. Ayouba, E. Nerrienet, H. M. McClure, J. L. Heeney, A. E. Pusey, D. A. Collins, C. Boesch, R. W. Wrangham, J. Goodall, P. M. Sharp, G. M. Shaw, and B. H. Hahn.** 2002. SIVcpz in wild chimpanzees. Science **295:**465.

324. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J Exp Med **174:**407-15.

325. **Sawyer, S. L., L. I. Wu, M. Emerman, and H. S. Malik.** 2005. Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. Proc Natl Acad Sci U S A **102:**2832-7.

326. **Schienman, J. E., R. A. Holt, M. R. Auerbach, and C. B. Stewart.** 2006. Duplication and divergence of 2 distinct pancreatic ribonuclease genes in leaf-eating African and Asian colobine monkeys. Mol Biol Evol **23:**1465-79.

327. **Schols, D., and E. De Clercq.** 1998. The simian immunodeficiency virus mnd(GB-1) strain uses CXCR4, not CCR5, as coreceptor for entry in human cells. J Gen Virol **79 ( Pt 9):**2203-5.

328. **Schrofelbauer, B., D. Chen, and N. R. Landau.** 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). Proc. Natl. Acad. Sci. USA **101:**3927-3932.

329. **Schwartlander, B., G. Garnett, N. Walker, and R. Anderson.** 2000. AIDS in a new millennium. Science **289:**64-6.

330. **Shah, K. V., H. L. Ozer, H. S. Pond, L. D. Palma, and G. P. Murphy.** 1971. SV40 neutralizing antibodies in sera of US residents without history of polio immunization. Nature **231:**448-9.

331. **Shanmugam, V., W. M. Switzer, J. N. Nkengasong, G. Garcia-Lerma, T. A. Green, E. Ekpini, M. Sassan-Morokro, F. Antunes, K. Manshino, V. Soriano, S. Z. Wiktor, and W. Heneine.** 2000. Lower HIV-2 plasma viral loads may explain differences between the natural histories of HIV-1 and HIV-2 infections. J Acquir Immune Defic Syndr **24:**257-63.

332. **Sharp, P. M., E. Bailes, R. R. Chaudhuri, C. M. Rodenburg, M. O. Santiago, and B. H. Hahn.** 2001. The origins of acquired immune deficiency syndrome viruses: where and when? Philos Trans R Soc Lond B Biol Sci **356:**867-76.

333. **Sharp, P. M., E. Bailes, F. Gao, B. E. Beer, V. M. Hirsch, and B. H. Hahn.** 2000. Origins and evolution of AIDS viruses: estimating the time-scale. Biochem Soc Trans **28:**275-82.

334. **Sharp, P. M., E. Bailes, D. L. Robertson, F. Gao, and B. H. Hahn.** 1999. Origins and evolution of AIDS viruses. Biol Bull **196:**338-42.

335. **Sharp, P. M., E. Bailes, M. Stevenson, M. Emerman, and B. H. Hahn.** 1996. Gene acquisition in HIV and SIV. Nature **383:**586-7.

336. **Sharp, P. M., and W. H. Li.** 1988. Understanding the origins of AIDS viruses. Nature **336:**315.

337. **Sharp, P. M., D. L. Robertson, F. Gao, and B. Hahn.** 1994. Origins and diversity of human immunodeficiency viruses. AIDS **8:**S27-S42.

338. **Sharp, P. M., G. M. Shaw, and B. H. Hahn.** 2005. Simian immunodeficiency virus infection of chimpanzees. Journal of Virology **79:**3891-902.

339. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646-50.

340. **Shibata, R., M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, and A. Adachi.** 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol **65:**3514-20.

341. **Shimada, M. K., K. Terao, and T. Shotake.** 2002. Mitochondrial sequence diversity within a subspecies of savanna monkeys (*Cercopithecus aethiops*) is similar to that between subspecies. J Hered **93:**9-18.

342. **Silvestri, G.** 2005. Naturally SIV-infected sooty mangabeys: are we closer to understanding why they do not develop AIDS? J Med Primatol **34:**243-52.

343. **Silvestri, G., A. Fedanov, S. Germon, N. Kozyr, W. J. Kaiser, D. A. Garber, H. McClure, M. B. Feinberg, and S. I. Staprans.** 2005. Divergent host responses during primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. J Virol **79:**4043-54.

344. **Silvestri, G., D. L. Sodora, R. A. Koup, M. Paiardini, S. P. O'Neil, H. M. McClure, S. I. Staprans, and M. B. Feinberg.** 2003. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. Immunity **18:**441-52.

345. **Simon, F., P. Mauclere, P. Roques, I. Loussert-Ajaka, M. C. Muller-Trutwin, S. Saragosti, M. C. Georges-Courbot, F. Barre-Sinoussi, and F. Brun-Vezinet.** 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat Med **4:**1032-7.

346. **Simon, F., S. Souquiere, F. Damond, A. Kfutwah, M. Makuwa, E. Leroy, P. Rouquet, J. L. Berthier, J. Rigoulet, A. Lecu, P. T. Telfer, I. Pandrea, J. C. Plantier, F. Barre-Sinoussi, P. Roques, M. C. Muller-Trutwin, and C. Apetrei.** 2001. Synthetic peptide strategy for the detection of and discrimination among highly divergent primate lentiviruses. AIDS Res Hum Retroviruses **17:**937-52.

347. **Slattery, J. P., G. Franchini, and A. Gessain.** 1999. Genomic evolution, patterns of global dissemination, and interspecies transmission of human and simian T-cell leukemia/lymphotropic viruses. Genome Res **9:**525-40.

348. **Sleeman, J. M., L. L. Meader, A. B. Mudakikwa, J. W. Foster, and S. Patton.** 2000. Gastrointestinal parasites of mountain gorillas (*Gorilla gorilla beringei*) in the Parc National des Volcans, Rwanda. J Zoo Wildl Med **31:**322-8.

349. **Smith, S. M., M. Makuwa, F. Lee, A. Gettie, C. Russo, and P. A. Marx.** 1998. SIVrcm infection of macaques. J Med Primatol **27:**94-8.

350. **Souquiere, S., F. Bibollet-Ruche, D. L. Robertson, M. Makuwa, C. Apetrei, R. Onanga, C. Kornfeld, J. C. Plantier, F. Gao, K. Abernethy, L. J. White, W. Karesh, P. Telfer, E. J. Wickings, P. Mauclere, P. A. Marx, F. Barre-Sinoussi, B. H. Hahn, M. C. Muller-Trutwin,**

**and F. Simon.** 2001. Wild *Mandrillus sphinx* are carriers of two types of lentivirus. J Virol **75:**7086-96.

351. **Stanford, C.** 1998. To catch a colobus. Prentice Hall, New Jersey, USA.

352. **Stanford, C. B., J. Wallis, H. Matama, and J. Goodall.** 1994. Patterns of predation by chimpanzees on red colobus monkeys in Gombe National Park, 1982-1991. Am J Phys Anthropol **94:**213-28.

353. **Starin, E. D.** 2006. Patterns of food transfer in Temminck's red colobus. Aggressive Behavior **32:**181-186.

354. **Starin, E. D.** 1994. Philopatry and affiliation among red colobus. Behaviour **130:**253-270.

355. **Starin, E. D.** 1989. Threats to the monkeys of The Gambia. Oryx **23:**208-214.

356. **Stern, M., and R. Goldstone.** 2005. Red colobus as prey: the leaping habits of five sympatric Old World monkeys. Folia Primatol (Basel) **76:**100-12.

357. **Sterner, K. N., R. L. Raaum, Y. P. Zhang, C. B. Stewart, and T. R. Disotell.** 2006. Mitochondrial data support an odd-nosed colobine clade. Mol Phylogenet Evol **40:**1-7.

358. **Stoorvogel, J. J.** 1993. Gross inputs and outputs of nutrients in undisturbed forest, Taï Area, Côte d'Ivoire. Veenman Drukkers, Wageningen.

359. **Stowell, R. E., E. K. Smith, C. Espana, and V. G. Nelson.** 1971. Outbreak of malignant lymphoma in rhesus monkeys. Lab Invest **25:**476-9.

360. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature **427:**848-53.

361. **Struhsaker, T. T.** 1981. Vocalizations, Phylogeny and Palaeogeography of Red Colobus Monkeys (*Colobus badius*). African Journal of Ecology **19:**265-283.

362. **Switzer, W. M., B. Parekh, V. Shanmugam, V. Bhullar, S. Phillips, J. J. Ely, and W. Heneine.** 2005. The epidemiology of simian immunodeficiency virus infection in a large number of wild- and captive-born chimpanzees: evidence for a recent introduction following chimpanzee divergence. AIDS Res Hum Retroviruses **21:**335-42.

363. **Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L. P. Waits, and J. Bouvet.** 1996. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Research **24:**3189-94.

364. **Takehisa, J., Y. Harada, N. Ndembi, I. Mboudjeka, Y. Taniguchi, C. Ngansop, S. Kuate, L. Zekeng, K. Ibuki, T. Shimada, B. Bikandou, Y. Yamaguchi-Kabata, T. Miura, M. Ikeda, H. Ichimura, L. Kaptue, and M. Hayami.** 2001. Natural infection of wild-born mandrills (*Mandrillus sphinx*) with two different types of simian immunodeficiency virus. AIDS Res Hum Retroviruses **17:**1143-54.

365. **Takehisa, J., L. Zekeng, E. Ido, Y. Yamaguchi-Kabata, I. Mboudjeka, Y. Harada, T. Miura, L. Kaptu, and M. Hayami.** 1999. Human immunodeficiency virus type 1 intergroup (M/O) recombination in cameroon. J Virol **73:**6810-20.

366. **Takemura, T., M. Ekwalanga, B. Bikandou, E. Ido, Y. Yamaguchi-Kabata, S. Ohkura, H. Harada, J. Takehisa, H. Ichimura, H. J. Parra, M. Nende, E. Mubwo, M. Sepole, M. Hayami, and T. Miura.** 2005. A novel simian immunodeficiency virus from black mangabey (*Lophocebus aterrimus*) in the Democratic Republic of Congo. J Gen Virol **86:**1967-71.

367. **Takemura, T., and M. Hayami.** 2004. Phylogenetic analysis of SIV derived from mandrill and drill. Front Biosci **9:**513-20.

368. **Tappen, M., and R. Wrangham.** 2000. Recognizing hominoid-modified bones: the taphonomy of colobus bones partially digested by free-ranging chimpanzees in the Kibale Forest, Uganda. Am J Phys Anthropol **113:**217-34.

369. **Taylor, L. H., S. M. Latham, and M. E. J. Woolhouse.** 2001. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci **356:**983-989.

370. **Telfer, P. T., S. Souquiere, S. L. Clifford, K. A. Abernethy, M. W. Bruford, T. R. Disotell, K. N. Sterner, P. Roques, P. A. Marx, and E. J. Wickings.** 2003. Molecular evidence for deep phylogenetic divergence in *Mandrillus sphinx*. Mol Ecol **12:**2019-24.

371. **Temin, H. M.** 1993. Retrovirus variation and reverse transcription: abnormal strand transfers result in retrovirus genetic variation. Proc Natl Acad Sci U S A **90:**6900-3.

372. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:**4876-82.

373. **Thompson, J. D., D. G. Higgings, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22** 4673-80.

374. **Ting, N.** 2008. Mitochondrial relationships and divergence dates of the African colobines: Evidence of Miocene origins for the living colobus monkeys. J Hum Evol (In Press).

375. **Tomonaga, K., J. Katahira, M. Fukasawa, M. A. Hassan, M. Kawamura, H. Akari, T. Miura, T. Goto, M. Nakai, M. Suleman, and et al.** 1993. Isolation and characterization of simian immunodeficiency virus from African white-crowned mangabey monkeys (*Cercocebus torquatus lunulatus*). Arch Virol **129:**77-92.

376. **Tosi, A. J., T. R. Disotell, J. C. Morales, and D. J. Melnick.** 2003. Cercopithecine Y-chromosome data provide a test of competing morphological evolutionary hypotheses. Mol Phylogenet Evol **27:**510-21.

377. **Traina-Dorge, V., J. Blanchard, L. Martin, and M. Murphey-Corb.** 1992. Immunodeficiency and lymphoproliferative disease in an African green monkey dually infected with SIV and STLV-I. AIDS Res Hum Retroviruses **8:**97-100.

378. **Trivers, R. L.** 1972. Parental investment and sexual selection., p. 136-179. *In* B. Campbell (ed.), Sexual Selection and the Descent of Man. Heinemann Educational Books Ltd., London.

379. **Tutin, C. E.** 2000. Ecology and social organization of African tropical forest primates: aid in understanding retrovirus transmission. Bull Soc Pathol Exot. **93:**157-61.

380. **UNAIDS** 2007, posting date. AIDS epidemic update: available on the web. [Online.]

381. **United Nations.** 2001. Report of the panel of experts on the illegal exploitation of natural resources and other forms of wealth of the Democratic Republic of Congo. United Nations, New York.

382. **van de Woude, S., and C. Apetrei.** 2006. Going wild: lessons from naturally occurring T-lymphotropic lentiviruses. Clin Microbiol Rev **19:**728-62.

383. **van der Kuyl, A. C., J. T. Dekker, and J. Goudsmit.** 2000. Primate genus Miopithecus:evidence for the existence of species and subspecies of dwarf guenons based on cellular and endogenous viral sequences. Mol. Phylogenet. Evol. **14:**403-413.

384. **Van Heuverswyn, F., Y. Li, E. Bailes, C. Neel, B. Lafay, B. F. Keele, K. S. Shaw, J. Takehisa, M. H. Kraus, S. Loul, C. Butel, F. Liegeois, B. Yangda, P. M. Sharp, E. Mpoudi-Ngole, E. Delaporte, B. H. Hahn, and M. Peeters.** 2007. Genetic diversity and phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon. Virology **368:**155-71.

385. **Van Heuverswyn, F., Y. Li, E. Bailes, C. Neel, B. Lafay, B. F. Keele, K. S. Shaw, J. Takehisa, M. H. Kraus, S. Loul, C. Butel, F. Liegeois, B. Yangda, P. M. Sharp, E. Mpoudi-Ngole, E. Delaporte, B. H. Hahn, and M. Peeters.** 2007. Genetic diversity and phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon. Virology.

386. **Van Heuverswyn, F., Y. Li, C. Neel, E. Bailes, B. F. Keele, W. Liu, S. Loul, C. Butel, F. Liegeois, Y. Bienvenue, E. M. Ngolle, P. M. Sharp, G. M. Shaw, E. Delaporte, B. H. Hahn, and M. Peeters.** 2006. Human immunodeficiency viruses: SIV infection in wild gorillas. Nature **444:**164.

387. **Van Heuverswyn, F., and M. Peeters.** 2007. The Origins of HIV and Implications for the Global Epidemic. Curr Infect Dis Rep **9:**338-346.

388. **van Hooff, J. A. R. A. M., and C. P. van Schaik.** 1992. Cooperation in competition: the ecology of primate bonds, p. 357-389. *In* A. H. Harcourt and F. B. M. de Waal (ed.), Coalition and alliances between humans and other animals. Oxford University Press, Oxford.

389. **van Oirschot, B.** 1999. Group fission in western red colobus (*Procolobus badius*) Taï National Park, Côte d'Ivoire. MsC Thesis. Universiteit Leiden, Leiden.

390. **van Rensburg, E. J., S. Engelbrecht, J. Mwenda, J. D. Laten, B. A. Robson, T. Stander, and G. K. Chege.** 1998. Simian immunodeficiency viruses (SIVs) from eastern and southern Africa: detection of a SIVagm variant from a chacma baboon. J Gen Virol **79 ( Pt 7):**1809-14.

391. **van Schaik, C. P.** 1989. The ecology of social relationships amongst female primates., p. 195-218. *In* V. Standen and R. A. Foley (ed.), Comparative Socioecology. The behavioural Ecology of Humans and Other Mammals. Blackwell, Oxford.

392. **Vergne, L., A. Bourgeois, E. Mpoudi-Ngole, R. Mougnutou, J. Mbuagbaw, F. Liegeois, C. Laurent, C. Butel, L. Zekeng, E. Delaporte, and M. Peeters.** 2003. Biological and genetic characteristics of HIV infections in Cameroon reveals dual group M and O infections and a correlation between SI-inducing phenotype of the predominant CRF02_AG variant and disease stage. Virology **310:**254-66.

393. **Verschoor, E. J., Z. Fagrouch, I. Bontjer, H. Niphuis, and J. L. Heeney.** 2004. A novel simian immunodeficiency virus isolated from a Schmidt's guenon (*Cercopithecus ascanius schmidti*). J Gen Virol **85:**21-4.

394. **Vidal, N., M. Peeters, C. Mulanga-Kabeya, N. Nzilambi, D. Robertson, W. Ilunga, H. Sema, K. Tshimanga, B. Bongo, and E. Delaporte.** 2000. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. J Virol **74:**10498-507.

395. **Vigilant, L.** 1999. An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. Biol Chem **380:**1329-31.

396. **Villesen, P., and T. Fredsted.** 2006. Fast and non-invasive PCR sexing of primates: apes, Old World monkeys, New World monkeys and Strepsirrhines. BMC Ecology **6:**8.

397. **Wain-Hobson, S.** 1992. Human immunodeficiency virus type 1 quasispecies in vivo and ex vivo. Curr Top Microbiol Immunol **176:**181-93.

398. **Wallis, J., and D. R. Lee.** 1999. Primate conservation: the prevention of disease transmission. International Journal of Primatology **20:**803-826.

399. **Warren, K. S., H. Niphuis, Heriyanto, E. J. Verschoor, R. A. Swan, and J. L. Heeney.** 1998. Seroprevalence of specific viral infections in confiscated orangutans (*Pongo pygmaeus*). J Med Primatol **27:**33-7.

400. **Watts, D. P., J. C. Mitani, and H. M. Sherrow.** 2002. New cases of inter-community infanticide by male chimpanzees at Ngogo, Kibale National Park, Uganda. Primates **43:**263-70.

401. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, and et al.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117-22.

402. **Wertheim, J. O., and M. Worobey.** 2007. A challenge to the ancient origin of SIVagm based on African green monkey mitochondrial genomes. PLoS Pathog **3:**866-873.

403. **Westmoreland, S. V., E. Halpern, and A. A. Lackner.** 1998. Simian immunodeficiency virus encephalitis in rhesus macaques is associated with rapid disease progression. J Neurovirol **4:**260-8.

404. **Whelan, S., and N. Goldman.** 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol Biol Evol **18:**691-699.

405. **Whitesides, G. H.** 1989. Interspecific associations of Diana monkeys, *Cercopithecus diana*, in Sierra Leone, West Africa, Biological significance or chance? Animal Behaviour **37:**760-776.

406. **Wilkie, D.** 2006. Bushmeat: a disease risk worth taking to put food on the table? Animal Conservation **9** 370-371.

407. **Wilkie, D., and J. Carpenter.** 1999. Bushmeat hunting in the Congo Basin: an assessment of impacts and options for mitigation. Biodiversity and Conservation **8:**137-147.

408. **Wilkie, D., E. Shaw, F. Rotberg, G. Morelli, and P. Auzel.** 2000. Roads, development, and conservation in the congo Basin. Conser Biol **14:**1614-1622.

409. **Wilkie, D. S., J. G. Sidle, and G. C. Boundzanga.** 1992. Mechanized logging, market hunting, and bank loan in Congo. Conservation Biology **6:**1-11.

410. **Withmore, T. C.** 1990. An introduction to tropical rain forests. Clarendon Press, Oxford.

411. **Wolfe, N. D., A. A. Escalante, W. B. Karesh, A. Kilbourn, A. Spielman, and A. A. Lal.** 1998. Wild primate populations in emerging infectious disease research: the missing link? Emerg Infect Dis **4:**149-58.

412. **Wolfe, N. D., W. Heneine, J. K. Carr, A. D. Garcia, V. Shanmugam, U. Tamoufe, J. N. Torimiro, A. T. Prosser, M. Lebreton, E. Mpoudi-Ngole, F. E. McCutchan, D. L. Birx, T. M. Folks, D. S. Burke, and W. M. Switzer.** 2005. Emergence of unique primate T-lymphotropic viruses among central African bushmeat hunters. Proc Natl Acad Sci U S A **102:**7994-9.

413. **Wolfe, N. D., W. M. Switzer, J. K. Carr, V. B. Bhullar, V. Shanmugam, U. Tamoufe, A. T. Prosser, J. N. Torimiro, A. Wright, E. Mpoudi-Ngole, F. E. McCutchan, D. L. Birx, T. M. Folks, D. S. Burke, and W. Heneine.** 2004. Naturally acquired simian retrovirus infections in central African hunters. Lancet **363:**932-7.

414. **Wolfheim, J. H.** 1983. Primates of the world: distribution, abundance and conservation. University of Washington Press, Seattle.

415. **World Health Organization.** 2003. Outbreak(s) of Ebola haemorrhagic fever, October 2001-July 2002, p. 223-8, Wkly Epidemiol Rec.

416. **Worobey, M., M. L. Santiago, B. F. Keele, J. B. Ndjango, J. B. Joy, B. L. Labama, A. B. Dhed, A. Rambaut, P. M. Sharp, G. M. Shaw, and B. H. Hahn.** 2004. Origin of AIDS: contaminated polio vaccine theory refuted. Nature **428:**820.

417. **Wu, X. X., X. M. Tu, F. Q. He, H. J. Shi, Q. Wei, H. Jiang, and J. D. Shi.** 1991. Studies on the monitoring of viruses in Chinese rhesus monkeys (*Macaca mulatta*). Chin J Lab Anim Sci **1:**179-83.

418. **Yamaguchi, J., S. G. Devare, and C. A. Brennan.** 2000. Identification of a new HIV-2 subtype based on phylogenetic analysis of full-length genomic sequence. AIDS Res Hum Retroviruses **16:**925-30.

419. **Yang, B., and B. Rannala.** 1997. Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method. Mol Biol Evol. **14:**717-24.

420. **Yang, C., B. C. Dash, F. Simon, G. van der Groen, D. Pieniazek, F. Gao, B. H. Hahn, and R. B. Lal.** 2000. Detection of diverse variants of human immunodeficiency virus-1 groups M, N, and O and simian immunodeficiency viruses from chimpanzees by using generic pol and env primer pairs. J Infect Dis **181:**1791-5.

421. **Yang, Z., and N. Goldman.** 1994. [Evaluation and extension of Markov process models for the evolution of DNA]. Yi Chuan Xue Bao **21:**17-23.

422. **Yang, Z., R. Nielsen, and M. Hasegawa.** 1998. Models of amino acid substitution and applications to mitochondrial protein evolution. Mol Biol Evol **15:**1600-1611.

423. **Zander, R. H.** 2004. Minimal values for reliability of bootstrap and jacknife proportions, decay index, and bayesian posterior probabilities. PhyloInformatics **2:**1-13.

424. **Zhang, Y., B. Lou, R. B. Lal, A. Gettie, P. A. Marx, and J. P. Moore.** 2000. Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. J. Virol. **74:**6893-6910.

425. **Zhu, T., B. T. Korber, A. J. Nahmias, E. Hooper, P. M. Sharp, and D. D. Ho.** 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature **391:**594-597.

426. **Zuberbühler, K.** 2001. Predator-specific alarm calls in Campbell's guenons. Behavioral Ecology and Sociobiology **50:**414-422.

427. **Zuberbuhler, K., and D. Jenny.** 2002. Leopard predation and primate evolution. J Hum Evol **43:**873-86.

428. **Zuberbühler, K., R. Noë, and R. M. Seyfarth.** 1997. Diana monkeys long-distance calls: messages for conspecifics and predators. Animal Behaviour **53:**589-604.

# 14. Curriculum vitae

| | |
|---|---|
| **Name:** | Sabrina Locatelli |
| **Place, date of birth:** | Sorengo, Switzerland, 12th of December 1973 |
| **Nationality:** | Swiss |
| **Languages:** | Italian (mother tongue), French, English, Spanish and German |

**Education and work experience:**

| | |
|---|---|
| **1979-1992** | Primary, secondary and Highschool in Lugano, Switzerland |
| **1992-1997** | **Licence en Biologie**, Diplôme d'Etat. Faculté des Sciences, Université de Lausanne (VD), Switzerland. Mémoire de licence on: *'Using 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor superantigen-induced T cell division in vitro and in vivo'.* Supervision: Dr. T. Renno |
| **1997 - 1998** | **MSc in Immunology** Dipartimento di Farmacologia, Università degli Studi, Milano, Italy - Faculté des Sciences, Université de Lausanne (VD), Switzerland. MSc Thesis on *'Effects of Tramadol, an analgesic drug, on immune responses in rodents and humans'*. Supervision: Prof. A. Panerai, Prof. E. Farmer, and Prof. U. T.Ruegg |
| **1998** | **Volunteer for OFI (Orangutan Foundation International)**. Tanjung Puting National Park, Kalimantan Tengah (Borneo), Indonesia. Supervision: Prof. B. Galdikas |
| **1999** | **Internship in animal behavior and cognition** Chimpanzee and Human communication Institute' (CHCI), Central Washington University (USA). Supervision: Dr. R. Fouts |

| | |
|---|---|
| **2000** | **Assistant keeper, biological and behavioral data collector, zoo visitor guide** Zoological Garden Basel (Switzerland). Supervision: Dr. G. Guldenschuh |
| **2000 - 2001** | **MSc of Sciences in Primate Conservation** School of Social Sciences and Law, Oxford Brookes University, Oxford, UK. MSc report: *'Zoo visitors and primate conservation'*, in collaboration with the Zoological Garden, Basel (Switzerland). Supervision: Prof. S. K. Bearder and Dr. K. Hill |
| **2002** | **Study on social relationships among adult female chimpanzees.** Taï National Park, Côte d'Ivoire (*Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany.* Supervision: Prof. C. Boesch |
| **2003** | **Full time secondary school teacher (Sciences) and High school teacher (Biology) (short term contract)** Cadenazzo and Locarno, Switzerland<br>**Co-manager, camp administrator and project assistant (short contract).** Salonga National Park, Democratic Republic of Congo *(Max Planck Institut, Leipzig and Max Planck Institut, Seewiesen, Germany).* Supervision: Dr. B. Fruth and Dr. G. Hohmann |
| **2004 - 2008** | **PhD program in Public Health and Epidemiology.** Faculty of Science, University of Basel, Switzerland . PhD thesis: *Simian immunodeficiency virus molecular epidemiology in non-human primates from West Africa*. Supervision: Dr. M. Peeters (IRD, Montpellier) and PD Dr. J. Zinsstag (STI, Basel) |

**Funding:**

Kommission für Forschungspartnerschaften mit Entwicklungsländern (KFPE), Bern; Messerli Foundation, Zurich; Guggenheim-Schnurr Foundation, Basel, Switzerland; Jubilee Foundation, Basel, Switzerland; Institut de Recherche pour le Développement (IRD), Montepllier, France and the Agence Nationale de Recherches pour le SIDA (ANRS).

**Oral and Poster presentations at scientific meetings:**

**17th-20th Apr 07** 14th International Workshop on HIV Dynamics & Evolution, Segovia, Spain

"*Simian Immunodeficiency virus infection in wild-living red colobus monkeys from the Taï Forest in Côte d'Ivoire*"

**3rd-8th Sept 07** 2nd Conference of the European Society of Primatology, Prague, Check Republic

"*Prevalence and genetic diversity of simian immunodeficiency virus infection in wild-living red colobus monkeys, from the Taï Forest Côte d'Ivoire*"

**19th Nov 07** Seminar at Oxford Brooks University, Oxford, UK

"*Simian immunodeficiency virus and poaching pressure in the Taï Forest, Côte d'Ivoire: potential for a HIV-3 emergence?*"

**3rd-8th Aug 08** XXII Congress International Primatological Society , Edinburgh, Scotland.

"*Simian immunodeficiency virus (SIV) prevalence and molecular characteristics in western red colobus species* (Piliocolobus badius badius *and* Piliocolobus badius temminckii) *from the Taï National Park, Côte d'Ivoire and Abuko Nature Reserve, The Gambia*"

**List of publications:**

**1.** Renno T., Attinger A., **Locatelli S.**, Bakker T., Vacheron S., MacDonald HR (1999) 'Cutting edge: apoptosis of superantigen-activated T cells occurs preferentially after a discrete number of cell divisions in vivo', *Journal of Immunology*, **162** (11): 6312-5.

**2. Locatelli S**, Liegeois F, Lafay B, Roeder AD, Bruford MW, Formenty P, Noë R, Delaporte E, Peeters M (2008) 'Prevalence and genetic diversity of Simian Immunodeficiency virus infection in wild-living red colobus monkeys (*Piliocolobus badius badius*) from the Taï Forest, Côte d'Ivoire', *Infection Genetics and Evolution*, **8** (1): 1-14.

**3.** Liegeois F, Lafay B, Switzer WB, **Locatelli S**, Mpoudi-Ngolé E, Loul S, Delaporte E and Peeters M. (2008) 'Identification and molecular characterization of new STLV-1 and STLV-3 strains in non-human primates in Cameroon', *Virology*, **371** (2): 405-17.

**4. Locatelli S**, Lafay B, Liegeois F, Ting N, Delaporte E, Peeters M. (2008) 'Full molecular characterisation of a simian immunodeficiency virus, SIVwrc-*Pbt* from Temminck's red colobus (*Piliocolobus badius temminckii)* from the Abuko Nature Reserve, the Gambia',*Virology*. In Press.

**5. Locatelli S**, Roeder AM, Bruford MW (2008) 'Microsatellite markers in seven monkey species from Taï National Park, Côte d'Ivoire: application to non-invasively collected samples'. Submitted to *Folia Primatologica.*

**6.** Liegeois F, Lafay B, **Locatelli S**, Formenty P, Delaporte E and Peeters M. (2008) 'Full molecular characterisation of Simian immunodeficiency virus from western red colobus (*Piliocolobus badius badius*) and olive colobus (*Procolobus verus*) from the Taï Forest, Côte d'Ivoire'. In preparation.